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MAMMALIAN SOMATIC CELL HYBRIDIZATION

BY

© JARLEY KOO

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled:

MAMMALIAN SOMATIC CELL HYBRIDIZATION

Submitted by: JARLEY KOO

in partial fulfilment of the requirements for the degree of M.Sc.

".....The problem of organization is the central problem of biology.....the riddle of form is the fundamental riddle".

J. Needham, Order and Life.

TO MY PARENTS

ABSTRACT

That mammalian somatic cell hybridization is a well established investigative tool in the genetic analysis of somatic cells is beyond dispute. Recently it has been clearly demonstrated that this cell fusion phenomenon may play a very important biological role in the In Vivo behaviour of malignant tumours. Among the interspecific hybrids, the human-mouse combination is the most studied. However, little has been said of the pattern of segregation of the human chromosomes and the factors influencing this selection in these hybrids.

The present investigation was undertaken to determine whether there is a definitive pattern of human chromosome retention in a human x mouse somatic hybrid. In Vitro human-mouse somatic cell hybrids were produced by fusing L5178Y mouse lymphoblasts with human embryonic fibroblasts using commercially available inactive "Sendai" virus. Karyotype studies of the resultant hybrid cell were carried out over a period of several months. Two distinct hybrid populations were produced; one in suspension resembling the mouse parent - the "S" cells; and the other in monolayer resembling the human fibroblasts- the "M" cells.

These two hybrid populations emerged at different times after the initial fusion reaction and with different frequencies. The karyotype of the "S" cells was analyzed in detail. Marked segregation

of human chromosomes occurred from these hybrid cells as is typical of other human-mouse hybrids. In this experimental model, human chromosome of the "A" group appear to be preferentially retained by the hybrid cells. By associating the In Vitro and In Vivo behaviour of the hybrid cells with the chromosomes of the "A" group, these experiments may add information regarding the influence of specific human genetic material on tumour cell morphology and behaviour.

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INTRODUCTION

Mammalian somatic cell hybridization provides an excellent biological model for genetic analysis. By this technique genetic material, either from the same species or from widely different species, can be introduced into the nuclei of somatic cells to produce hybrid cells which are capable of prolonged survival and replication.

This study is concerned with the pattern of chromosome segregation in a human X mouse hybrid population. Human chromosomes of the "A" group seem to be preferentially retained in our model. The factors producing this specific chromosomal segregation remain to be determined.

Literature Review.

I. History of Mammalian Somatic Cell Hybridization.

In 1960, Barski, Sorieul and Cornefert described for the first time the occurrence of spontaneous fusion of somatic cells in mixed culture and provided karyological evidence of the phenomenon of somatic cell hybridization In Vitro. (Barski et al, 1960; 1961; Barski and Cornefert, 1962). In their experiments, two cell lines, the high cancer N1 line derived from the NCTC 2475 clone and the low cancer N2 line from the NCTC 2555 clone (both originally cultured from the C3H mouse fibroblasts) were grown together in mixed culture. By the third month, a new cell type, designated M, emerged and reached a proportion of nearly one third by the eighth month. This new cell type was identified karyologically as a hybrid line; the cells contained approximately the sum of the parental chromosome numbers and a marker chromosome from the N1 cells. The M cells were subsequently cloned In Vivo and In Vitro: 15 clones were isolated and studied. It was found that in some features the M cells were intermediate between the two parent cell lines and in others, they resembled either one or the other parent. Thus, morphologically in culture, they shared characteristics with both the parental cells. On the other hand, their malignant potential, as measured by percentages of takes, rapidity of tumour growth In Vivo and the minimal take-dose in C3H mice, approximated that of the high cancer N1 line. Unexpectedly, however, the histological feature of the tumour produced

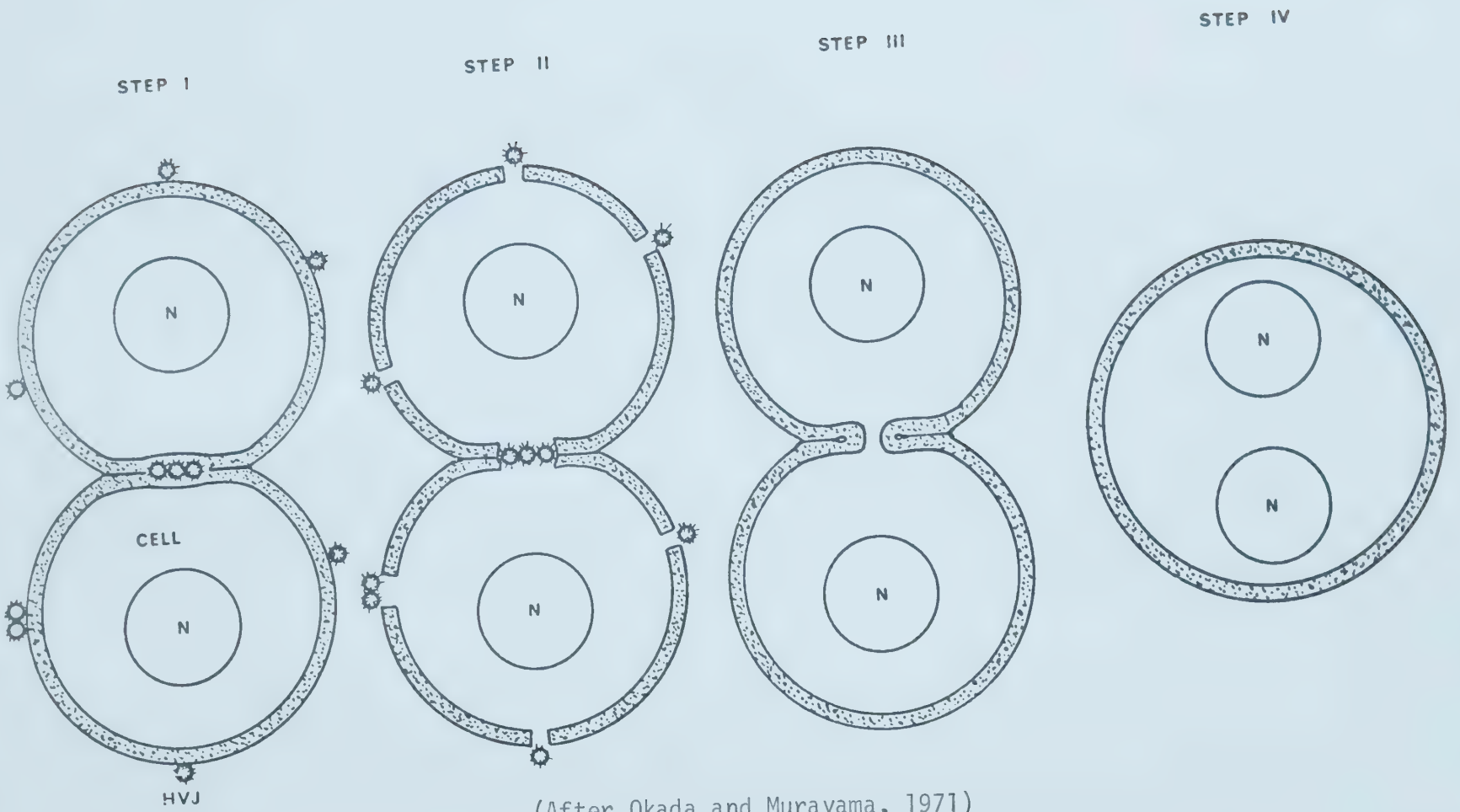
by the M cells was distinctly different from both the parental tumours. The M tumours consisted of irregularly arranged, large, round cells whereas the NI tumours were of the spindle-cell sarcoma variety and the N2 tumours were composed of anaplastic small round cells. The mean nuclear size of the M tumour cells was significantly larger than that of either NI or N2 tumour cells. In addition, Barski et al, found that during the 12 months study, the M cells were stable in their characteristics except that they tended to lose chromosomes with time and that a wider scatter of chromosome range became progressively more pronounced. From these results, they concluded that malignancy of a cell line is a dominant characteristic and that additional genetic material was probably responsible for cancerous transformation rather than the elimination of regulatory genes.

It was not long before Sorieul and Ephrussi were able to show that somatic cell hybridization observed by Barski et al was not a unique event. (Sorieul and Ephrussi, 1961). Ephrussi et al successfully hybridized cells from many different strains of mice and obtained viable hybrid progeny. (Ephrussi and Sorieul, 1962a; 1962b; Ephrussi et al, 1963; 1964; Ephrussi, 1965). Gershon and Sachs confirmed the results of Ephrussi et al by independent experiments. (Gershon and Sachs, 1963). However, in all these experiments, success depended on the hybrid cells having a selective growth advantage over

the parental cells so that the latter would be overgrown eventually. This factor is not predictable in advance.

II. Selective System for Isolation of Hybrid Cells.

In 1964, Littlefield reported a selective system for the isolation of hybrid cells in mixed culture. He selected two clonal sub-lines of L-cells derived from mouse fibroblasts, one lacking guanylic-acid-inosinic-acid pyrophosphorylase and the other thymidine kinase, thereby resistant to 3 ug/ml of 8-azaguanine and 30 ug/ml of 5-bromodeoxyuridine (BUdR) respectively. The two cell lines were grown in mixed culture in a medium to which 3×10^{-6} M. glycine, 1.6×10^{-5} M. thymidine, 1×10^{-4} M. hypoxanthine and 4×10^{-7} M. aminopterin were added, the last inhibiting the endogenous biosynthesis of purines and thymidylic acid. Only the hybrid cells would grow in this selective medium as they alone contained the necessary complimentary enzymes, derived respectively from the two parent cell lines, to make use of the hypoxanthine and thymidine in the medium. (Littlefield, 1963; 1964a; 1964b; 1965). Littlefield established that approximately 1 to 5 hybrid cells were produced per 1×10^{6} parental cells. (Littlefield, 1966). Davidson and Ephrussi modified Littlefield's selective technique and used a half selective system in which only one parental cell line was enzyme-deficient and inhibited by aminopterin; the other cell strain was a normal one, freshly explanted



(After Okada and Murayama, 1971)

from skins of newborn CBA mice carrying the T-6 translocation. (Davidson and Ephrussi, 1965). Hybrid cells grew rapidly as colonies on a background of CBA mouse fibroblast monolayer and were obtained with equal facility as in Littlefield's system.

III. Virus-Induced Cell Fusion.

In parallel development, Okada observed that Sendai virus (Synonym Hemagglutinating Virus of Japan, HVJ), a myxovirus, causes In Vitro clumping and fusion of Ehrlich's ascites tumour cells to form multinucleate giant cells. (Okada, 1958; 1961a). In a series of meticulous experiments, Okada et al examined the mechanisms of cell fusion by Sendai virus and defined the experimental conditions for optimal fusion reaction. (Okada, 1961b; Okada and Tadokoro, 1962; Okada, 1962; Okada and Tadokoro, 1963; Okada et al, 1964). The fusion reaction proceeds along the following steps: (Schneeberger and Harris, 1966; Okada and Murayama, 1961; Hosada and Koshi, 1968)

1. Agglutination of cells by virions in the cold (4°C).
2. Close contact of adjacent cell membranes of agglutinated cells at sites of absorbed virions.
3. Breakage of cell membranes at sites of viral absorption and direct communication of cytoplasm of adjacent cells.

4. Reconnection of the broken cell membranes and formation of cytoplasmic bridges at 37°C resulting in polynuclear giant cells.

Okada et al found that the viral-induced cell fusion reaction is an active process which requires the expenditure of energy and calcium ions (Okada et al, 1966; Okada and Murayama, 1966). To measure the efficiency of cell fuison, Okada and Tadokoro introduced the concept of fusion index, F.I., which is the ratio of the number of cells after virus-induced fusion to that in the control tube, less one. (Okada and Tadokoro, 1962). It was found that the fusion efficiency varies with the cell lines or strains used. Undifferentiated cells of established culture seem to have high fusion capacity, while differentiated cells of short-term culture have relatively low capacity. There is evidence to suggest that older cells have a higher fusion index than the younger ones of the same cell strain. The fusion index of each cell line or strain increases with the amount of virus imput until a maximum value is reached, then it declines with further increase of virus titre. (Okada and Murayama, 1965).

From their experimental data, Okada and Tadokoro were able to calculate that approximately 1300 absorbed virus particles per cell were required for a 50 percent decrease of cell number (F.I. 1.0.) (Okada and Tadokoro, 1962).

IV. Study of Artificial Heterokaryons.

In 1965, Harris et al began to systematically experiment with virus-induced somatic cell fusion. Sendai virus was inactivated by exposure to ultraviolet light to reduce its infectivity by a factor of 10^6 without interfering in its fusion capacity (Harris and Watkins, 1965). Using this method, Harris et al were able to induce formation of multinucleate cells which contained varying numbers of nuclei from both parental cells. The degree of multinuclearity of fused cells is proportionally related to the concentration of virus and the quantity and proportion of the parental cells used. Thus, controlled production of these artificial heterokaryons was ingeniously exploited for the study of nucleo-cytoplasmic interactions and genetic regulation in mammalian cells. (Harris, 1966; 1970; 1971). Using autoradiographic technique, Harris et al demonstrated that artificial heterokaryons of mammalian cells from different species are able to synthesize RNA, protein and DNA just as in normal animal cells. By hybridizing differentiated with undifferentiated cell lines, Harris et al established the following principles: (Harris et al, 1966: Engel et al, 1969a).

(a) If either of the parent cells normally synthesizes RNA, then RNA synthesis will take place in both types of nuclei in the heterokaryons, even if one of the parent cells normally does not synthesize RNA.

(b) If either of the parent cells normally synthesizes DNA, then DNA synthesis will take place in both types of nuclei in the heterokaryons, even if one of the parent cells normally does not synthesize DNA.

(c) If neither of the parent cells synthesizes DNA, then DNA synthesis will not take place in the heterokaryons.

In every case, the active cell initiates the synthesis of nucleic acids in the inactive partner. In no case the inactive cell suppresses synthesis in the active partner. Thus, when a mature hen erythrocyte nucleus, which does not normally synthesize either RNA or DNA, is introduced into the cytoplasm of a HeLa cell or a mouse fibroblast, it is reactivated: its volume increases and nucleic acids synthesis is resumed within 24 hours after fusion (Bolund et al, 1969). However, appearance of either the hen-specific surface antigens or hen erythrocyte-nucleus-induced enzymes is delayed until the erythrocyte nuclei develop visible nucleoli, a process which usually takes four to six days (Harris et al, 1969; Harris and Cook, 1969; Cook, 1970). The nucleolus, therefore, plays a critical role in the transfer of RNAs from the genes to the cytoplasms of the cell. There is no mandatory coupling between gene transcription and translation.

Unlike the synthesis of RNA and protein, synthesis of DNA is a discontinuous process which takes place only at one particular stage in the life cycle of a cell. In most of the multinucleate heterokaryons, only some of the nuclei in the cell synthesize DNA

and enter mitosis, while others remain in interphase. In a small proportion of heterokaryons, DNA synthesis become progressively synchronized and most or all of the nuclei enter mitosis synchronously (Johnson and Harris, 1969a; 1969b; Yamanaka and Okada, 1966; Rao and Johnson, 1970). However, the nuclear spindle usually fails to form and cell division does not occur, resulting in a single large mononucleate cell. A synkaryon is thus formed; homosynkaryon, where the original nuclei are of the same kind, the heterosynkaryons, where they are of different kind. Furthermore, synchronization of DNA synthesis is not an inevitable event in heterokaryons. For instance, when cells in interphase are fused with cells in mitosis, premature condensation of interphase chromosomes occurs. (Johnson and Rao, 1970; Johnson et al, 1970). Also, in some cases of heterokaryons, depending upon the parent cell types, the parental nuclei compete with each other for some cytoplasmic activating factor(s) essential for DNA synthesis. Instead of synchrony of DNA sunthesis and mitosis, anti-synchrony was observed in these heterokaryons. (Johnson and Harris, 1969c).

The majority of the heterokaryons and synkaryons continue to synthesize DNA. The rate of synthesis, however, falls progressively over several days, and eventually synthesis ceases. (Harris and Watkins, 1965). They seem incapable of continued propagation to give rise to a viable progeny (Harris et al, 1965). Only multinucleate cells containing initially a small number of nuclei are

able to multiply continuously under favourable culture conditions (Harris and Watkins, 1965; Harris et al, 1966). The di-hetero karyons and synkaryons containing one nucleus or chromosome set respectively from each parent seem to have the greatest potential for viable existence and continued replication; they usually dominate in hybrid cultures (Yamanaka and Okada, 1968; Engel et al, 1969b).

Perhaps the most important conclusion to be drawn from this group of experiments is the fact that cells from different species of vertebrates are compatible with each other when they are amalgamated into a single unit. It appears that in the cells of vertebrates there is, in general, no intracellular mechanism for the recognition of incompatibility similar to those responsible for the recognition and destruction of tissue or organ grafts exchanged between different individuals. It would seem that instructions from the genes of each of the species in heterokaryons are correctly translated by both sets of cytoplasmic components and that signals emanating from the hybrid cytoplasm are correctly read by the genes of any other species. This implies either that regulatory genetic loci throughout the vertebrate subphylum have a low order of specificity and operate predominantly in a "positive , turning-on" sense, or that the regulatory mechanisms which control the synthesis of specific proteins are epigenetic, operating mainly in the cell cytoplasm. All restriction on nucleic acid synthesis imposed by the

process of differentiation are reversible (Harris, 1966; Jacobson, 1968).

V. Selective System vs Sendai Virus Technique.

In 1966, Yerganian and Nell reported the first successful production and isolation of virus-induced hybrid cells capable of prolonged multiplication, thus demonstrating that the viral technique is just as applicable as Littlefield's selective system to somatic cell hybridization (Yerganian and Nell, 1966). Moreover, the viral technique has definite advantages over the selective system. The latter depends upon the introduction of specific mutations into the cells to be crossed. In mammalian cells this is a difficult and time-consuming process, and for some kinds of cells, it may be impossible. In addition, during the time it takes to select cells with the required enzyme deficiency, other changes may have occurred altering those very cell properties under study. Coon and Weiss were able to show that virus-induced hybrid cells have essentially similar properties to those which arose by spontaneous hybridization in Selective medium. The former appeared, however, 100 to 1000 times more frequently. (Coon and Weiss, 1969; Murayama and Okada, 1970). Virus-induced hybridization can be achieved with high efficiency between almost any two cell populations and without regard to laborious selective procedures. The resulting hybrid cells

can then be isolated by other cell culture techniques. Controlled production of somatic cell hybrids by the viral technique is feasible. (Klebe et al, 1970). On the other hand, the selective system provides a simple method of isolating hybrid colonies. Often, when enzyme-deficient cell lines are available, the two methods are combined to obtain high yield of hybrid cells.

VI. Intraspecific and Interspecific Somatic Cell Hybridization.

The first interspecific somatic cell hybridization was successfully performed by Ephrussi and Weiss in 1965 (Ephrussi and Weiss, 1965). Using the half-selective system, they were able to obtain andisolate rat-mouse interspecific hybrid cells which had distinct morphological features and contained chromosome components from both parents. (Weiss and Ephrussi, 1966a; 1966b). Many workers have since succeeded in hybridizing both intraspecific and interspecific cell lines or strains, using either Littlefield's selective system, the Sendai viral technique, or a combination of both methods. For instance, intraspecific somatic cell hybrids of mouse, (Yoshida and Ephrussi, 1967; Engel et al, 1968; Engel et al, 1969b; Ruddle et al, 1970), Syrian Hamster, (Marin and Littlefield, 1968; Marin, 1969; Littlefield, 1969), Chinese Hamster, (Kao et al, 1969; Chasin, 1972), and human (Silagi et al, 1969; Siniscalco et al, 1969; Simoni and de Carli, 1970; Nadler et al, 1970;

Schneider, 1973). Interspecific hybrids between rat and mouse, (Sonnenschein et al, 1968; Davidson and Benda, 1970; Schneider and Weiss, 1971), mouse and Chinese hamster, (Scalletta et al, 1967; Koyama et al, 1970; Handmaker, 1971), mouse and Syrian hamster, (Davidson et al, 1966; Migeon, 1968), Chinese and Armenian hamsters, (Yerganian and Nell, 1966; Sonnenschein et al, 1969), hamster and man, (Kao and Puck, 1970; Goldberg et al, 1971; Westerveld et al, 1971; van Someren et al, 1972; Westerveld and Meera Khan, 1972), monkey and mouse, (Jacobson, 1968; Cassingena et al, 1971), human and mouse,(Weiss and Green, 1967; Matsuya et al, 1968; Migeon and Miller, 1968; Nabholz et al, 1969; Matsuya and Green, 1969; Boone and Ruddle, 1969; Klebe et al, 1970; Santachiera et al, 1970; Ruddle et al, 1970; Kusano et al, 1971; Ruddle et al, 1971; Green et al, 1971; Miller et al, 1971; Grzeschik et al, 1971; Shows, 1972; Ruddle, 1973) human and rat, mink and bovine (Teplitz et al, 1968), and even human and mosquito (Zepp et al, 1971). Experiments of this kind have been continuously adding to our knowledge of the organization of mammalian somatic cells: the interactions of genes, the regulation and expression of differentiated and undifferentiated functions of mammalian cells, linkage relationship of genes, the mechanism of viral transformation, etc. It is now reasonable to expect that the mapping of the human chromosomes by means of human-mouse hybrids will be achieved in the not too distant future.

VIII. Characteristics of Hybrid Cells.

(i) General.

The Phenotype of the hybrid cells is the result of action and interaction of the active genes of both parents. As a result, the cellular features of the hybrid cells, such as cellular and colony morphology, growth rate, etc., might be expected to be, and often are, intermediate between the two parental cell types. (Migeon and Childs, 1970). This is, however, by no means the rule. Different hybrid clones resulting from the same cross often exhibit considerable variation in their characteristics, many hybrid clones having features which resemble neither parents.

(ii) Evolution of Hybrid Karyotype.

In intraspecific crosses, the initial karyotype of a hybrid line usually contains the sum or slightly less of the total number of chromosomes of each parent cell involved in its production. With successive generations, however, the hybrid cells tend to lose some of their chromosomes. This keryotypic instability is especially marked in the young hybrid cells. The rate and amount of loss of chromosomes are related to the parent cell types used. As many as ten to 30 percent of the initial number of chromosomes may be lost

during the first year of culture. (Ephrussi and Sorieul, 1962a; 1962b; Ephrussi et al, 1963; 1964; Ephrussi, 1965; Ephrussi and Weiss, 1969; Yoshida and Ephrussi, 1969). Indeed, Engel et al observed that their intraspecific mouse hybrid populations contained an increasing number of segregants, in which nearly a complete parental chromosomal set was lost so that the segregants' karyotype was both quantitatively and qualitatively very similar to those of the parental cells. (Engel et al, 1969b). On the other hand, some intraspecific hybrids, after the initial loss of chromosomes, become relatively stable in their karyotype during prolonged culture. (Ephrussi et al, 1963; 1964; Ephrussi, 1965; Ruddle, 1970).

Amongst the interspecific hybrids, the karyotypes of the hybrid cells like those of the intraspecific hybrids, are unstable, especially in the first few months of culture. These hybrids often display extreme scatter in their chromosome range. Although most interspecific hybrids, when first karyotyped, have chromosome numbers considerably less than the sum total of the parental chromosome complements, some hybrids were found to have chromosome numbers greater than the expected parental sum (Migeon, 1968; Koyama et al, 1970; Handmaker, 1971). This is due to fusion of one cell from one parent with a 2s cell, or with two or more cells, from the other parent. Even in this type of hybrids, however, segregation of chromosomes is evident. The extent and rate of chromosome loss from interspecific hybrids is

not random, but preferential: the chromosomes of one parent being lost to a significantly greater extent. Thus, in rat-mouse, Syrian or Chinese* - Hamster-mouse, monkey-mouse, and human-mouse or human-hamster hybrids, the rat, mouse, monkey and human chromosomes respectively are preferentially lost. (Ephrussi and Weiss, 1965; Migeon, 1968; Scalletta et al, 1967; Cassinsena et al, 1971; Weiss and Green, 1967). In the rat-mouse combination, chromosome segregation proceeds against the rat acrocentric and small metacentric chromosomes. In the hamster-mouse hybrids, the mouse telocentric chromosomes seem to be preferentially lost, although the loss of hamster acrocentric and small metacentric chromosomes is also significant. Interestingly enough, it was observed that in the rat-mouse and hamster-mouse combinations, the large and medium-sized metacentric chromosomes of mouse and hamster cells respectively increased significantly during culture. In some of these cases, this increase paralelled numerically the loss of mouse telocentric chromosomes, suggesting that the former could have arisen by centromere misdivision or centric fusion of the mouse telocentric chromosomes (Weiss and Ephrussi, 1966a).

(iii) Human-Mouse Somatic Hybrid Cells.

The first human and mouse somatic hybrid was isolated by Weiss and Green, (Weiss and Green, 1967). In this type of hybrid

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* In Chinese hamster-mouse hybrids, some recent authors have observed preferential loss of hamster chromosomes (Koyama et al, 1970; Handmaker, 1971).

cells, the preferential loss of human chromosomes is both rapid and extreme, amounting to at least 75%, and in some cases more than 95 percent of the human chromosome complements. The Human-Mouse hybrid cells contain anything from one to 45 human chromosomes, but nearly all, and in some cases, double the amount of the expected mouse chromosomes. When aneuploid human cell lines are used in hybridization, more human chromosomes are retained and the rate of chromosome loss is correspondingly slower. (Matsuya and Green, 1969). In contrast, fusion with diploid human cell strains rarely yield hybrid clones containing more than 15 human chromosomes. In some hybrid populations, a variable proportion of hybrid cells eventually cease to contain any identifiable human chromosome altogether and their karyotypes revert back to those of their mouse parent (Migeon and Miller, 1968; Matsuya et al, 1968).

IV. Type of Human Chromosomes retained in Human-Mouse Hybrid Cells.

Immediately after fusion, the human-mouse hybrids probably contain the full genetic complements of both parents. Rapid and extensive loss of human chromosomes then follows. In the presence of selective pressure, specific human chromosomes are retained in the hybrid cells. For instance, when a thymidine-kinase deficient

mouse line is used in hybridization, the resultant hybrid cells, which have survived in the selective medium, are found to contain consistently one or more human chromosomes belonging to the E group (Migeon and Miller, 1968; Matsuya et al, 1968; Migeon et al, 1969; Green et al, 1971). Hybrid clones which contained a single human E chromosome have been isolated. Analysis of the nature of thymidine kinase in this type of hybrid clones has allowed the assignment of the thymidine-kinase gene to a chromosome of the E group. In a similar manner, the human X chromosome was shown to contain the genes for hypoxanthine-guanine phosphoribosyl transferase (HGPRT), glucose-6-phosphate dehydrogenase (G6PD), and phosphoglycerate kinase (PGK) (Nabholz et al, 1969; Miller et al, 1971; Ruddle et al, 1971). An acrocentric chromosome of either the G or D group contains the gene for adenine phosphoribosyl transferase (Kusano et al, 1971).

In the absence of selective pressure, the loss of human chromosomes from human-mouse hybrids has been assumed to be random. However, Marin has observed that, in his intraspecific hamster hybrid, the loss of chromosomes took place in oriented groups (Marin, 1969). There is indirect evidence that this may be true for interspecific human-mouse hybrids as well. (Nabholz et al, 1969; Grzeschik et al, 1972). In these cases, it is likely that unknown selective processes or genic interaction are in operation.

(v) Differentiated vs. Undifferentiated Functions in Hybrid Cells.

It is difficult to generalize on the expression of differentiated functions in hybrid cells resulting from the fusion of a differentiated cell line with an undifferentiated one. It appears that in most cases the differentiated functions of one parent are extinguished or markedly reduced upon hybridization with an undifferentiated partmer, whether intraspecific or interspecific. Examples are: melanin synthesis (Davidson et al, 1966; 1968; Davidson and Yamamoto, 1968; Davidson, 1969), growth hormone synthesis (Sonnenschein et al, 1968), formation of differentiated tissues (Finch and Ephrussi, 1967), synthesis of ribosomal RNA (Eliceiri and Green, 1969), immunoglobulin synthesis (Periman, 1970; Mohit and Fan, 1971; Coffino et al, 1971), synthesis and induction of Tyrosine amino-transferase (Schneider and Weiss, 1971), Glycerol-3-phosphate dehydrogenase synthesis, protein S100 synthesis and the induction of lactate dehydrogenase (Davidson and Benda, 1970), Es-2 esterase activity (Klebe et al, 1970). In other cases, the activity of a cytoplasmic product was not totally suppressed in the hybrids but reduced to an intermediate level between the two parents; for instance, the activity of folate reductase (Littlefield, 1969), collagen and hyaluronic acid synthesis (Green et al, 1966). Furthermore, there are striking examples where highly differentiated cellular activities are retained or even enhanced

upon fusion with an undifferentiated partner. Thus many neural properties, such as electrically active membranes, acetycholin-esterase synthesis and neurite formation, are retained undiminished in the hybrid cells (Minna et al, 1971; 1972). Interferon production was found to be enhanced upon hybridization, and in one case, the quantity produced was ten times that of the parent hamster (Guggenheim et al, 1968; Carver et al, 1968).

(vi) The Expression of Malignancy in Somatic Hybrids.

In their original experiment of somatic cell hybridization, Barski et al observed that the hybrid clones gave rise to malignant tumours in C3H mice as frequently as the high cancer N1 line. (Barski et al, 1962). Gershon and Sachs tested the malignant potential of their hybrid cells in F_1 hybrid mice and confirmed the finding of Barski et al (Gershon and Sachs, 1963). Scalletta and Ephrussi observed that hybrids between the neoplastic cell line, NCTC 2472, and normal fibroblasts from CBA mice are neoplastic for both the C3H mice and the F_1 (C3H x CBA) hybrid mice (Scalletta and Ephrussi, 1965). It was concluded that malignancy of cells is a "dominant" characteristic. Furthermore, Defendi et al found that the In Vivo tumourigenicity of their polyoma-transformed Py 27-6 and P7 27-6-8 clones was enhanced upon hybridization with normal diploid mouse fibroblasts (Defendi et

al, 1967). However, upon subcloning of cancerous hybrid clones, hybrid clones were obtained which were markedly less cancerous or even non-malignant. This indicates the hybrid cells were extremely heterogenous with respect to tumourigenecity. Silagi hybridized a malignant melanoma cell line (B16) from C57BL mice with A9 azagunaine-resistant fibroblasts derived from C3H mice and observed the behaviour of the resultant hybrid cells (Silagi, 1967). She found that the hybrid cells failed to grow as solid tumours in either of the parental strain of mice, but five of the six hybrid clones grew as solid tumours in the F_1 (C3H x C57BL)hybrid mice, giving a total of 18 positive results out of 58 mice injected with the hybrid cells. Here again, the hybrid clones displayed wide scatter of malignant potential, ranging from clone A having a 60 percent take-incidence to clone D with no take at all in 11 mice tested. Karyological analysis of one tumour produced by the hybrid cells showed a range of chromosomes between 101 and 113 with a mode of 106; the calculated range is 102-129 with a mode of 110. In addition all hybrid clones had a high degree of contact inhibition, a characteristic of the A9 parent, suggesting that there is no inevitable association between malignant behaviour and the lack of contact inhibition. Weiss et al also found that contact sensitivity was retained in the hybrids between a contact-sensitive and a contact-insensitive cell line (Weiss et al, 1968).

Using the Erhlich ascites, the SEWA and the MSWBS tumour cell lines as the malignant partners and the A9 mouse fibroblasts as the non-malignant partner, Harris and Klein reported that the hybrid clones regularly failed to grow in the great majority of the F_1 hybrid test animals and that the low incidence of tumour growth was comparable to that obtained with A9 cells alone (Harris et al, 1969). This finding is in contradistinction from all previous workers (Ephrussi et al, 1969; Harris and Klein, 1969; Barski, 1970). In addition, in the few transplantable ascites tumours which developed late in mice after receiving the Ehrlich-A9 hybrid cells intraperitoneally, Karyotype analysis showed a marked reduction in chromosome number, (40 out of 130 chromosomes were lost), including at least ten bi-armed chromosomes of A9 origin. These results led Harris et al to postulate that malignancy can be suppressed by cell fusion provided the hybrids retain the complete chromosome complements of the parental cells, and that loss of chromosomes from the non-malignant or less malignant partner was associated with a reversion to malignancy. Subsequent experiments by the same group indicated that suppression of malignancy by cell fusion was not a property confined to the A9 fibroblasts. (Bregula et al, 1971; Wiener et al, 1971). Normal diploid mouse fibroblasts were found to have the same ability. Thus, contrary to most workers, Harris et al concluded that malignancy behaves as if it were a recessive character.

Additional observation was presented by Murayama and Okada who hybridized the Ehrlich ascites tumour cells and $L_{(ag)}$ fibroblasts, ddO and C3H being the respective hosts (Murayama and Okada, 1970). The Ehrlich ascites tumour cells regularly produce tumour in both the host mice, whereas the $L_{(ag)}$ cells are non-malignant. It was found that the uncloned hybrid cells failed to form tumour in both the ddO and C3H mice when injected subcutaneously, but intraperitoneal injection was effective in all animals tested, indicating the importance of the route of administration in tumour production. However, the 50 percent tumour producing dose of the hybrid cells was 10^4 higher than that of the Ehrlich ascites cells. Alternate passage of hybrid cells In Vitro and In Vivo did not affect their tumorigenicity. Karyological analysis of the hybrid-induced tumours gave a mean value of model number of chromosomes of 83 in ddO mice and 81 in C3H mice, the expected mode being 102, indicating approximately a 20 percent loss of chromosomes. Thus, the results of Murayama and Okada agreed with those of Harris et al.

Recently, Harris, Klein and Associates have provided strong evidence that mouse tumour cells are capable of fusing In Vivo with host cells (Wiener, 1972). This demonstration has very important implications. In Vivo cell fusion might be one of the mechanisms determining the character of malignant growth, or the spread of malignant growth. The "dominance" or "recessiveness" of malignancy

of tumour cells upon fusion with normal cells would have clear relevance in this regard. If In Vivo fusion with host cells can be shown to be a general phenomenon of malignant cell behaviour, it is obvious that this mechanism could provide a basis for greatly increased genetic variation in the growing tumour cell population, upon which selection can take place.

Objectives.

This research work is the first part of a larger project of mammalian somatic cell hybridization, which is designed with the following objectives:

1. To produce a mouse-human somatic cell hybrid.
2. To characterize the karyotype of the hybrid cells.
3. To determine if there is any pattern of preferential retention of human chromosomes in the hybrid cells.

Materials.

The decision to use mouse and human cells for the following hybridization experiments was made because: (a) mouse-human somatic hybrids have been produced with uniform success by many authors, and (b) these type of hybrids are particularly suitable for genetic analysis as the human chromosomes are preferentially segregated. The resultant hybrid clones which contain only one or two human chromosomes are excellent biological models to analyse the genetic influence of specific human chromosomes on cellular behaviour. Instead of using an established human cell line, normal diploid cell strains were used in this study because it was desired to study the effect of normal human genetic material on cancer cell behaviour.

I. L-5178Y Mouse Lymphoblast Cell Line.

The L-5178Y lymphoblast cell line was readily available in our laboratory. It was originally obtained from Dr. A.C. Satorelli of Yale University, through the courtesy of Dr. A.R.P. Patterson of the McEachern Research Laboratory, University of Alberta. This cell line has been maintained In Vivo in male BDF mice by intraperitoneal transplantation of 1×10^7 cells each week. In Vitro, the L-5178Y cells grow in suspension in spinner flasks in Fisher's Medium* for

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* All mediums and serum were purchased from Grand Island Biological Co. Grand Island, New York, 14072 and Berkeley, California.

Leukemic cells, supplemented with 15 percent horse serum*.

II. Normal Diploid Human Embryonic Fibroblasts.

Normal diploid human cells were obtained from three sources: human fetal kidneys, fetal amniotic fluid and infant foreskin. The human cells were grown in monolayer in Minimal Essential Medium* or Rosewell Park Memorial Institute Medium* (RPMI), both supplemented with 15 percent fetal calf serum*.

III. Sendai Virus (Syn. Hemagglutinating Virus of Japan, (HVJ).

Live Sendai Virus was purchased from the Microbiological Associated Inc., Bethesda, Maryland, and β-propriolactone-inactivated Sendai Virus from the Connaught Medical Research Laboratories, Willowdale, Ontario.

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* All mediums and serum were purchased from Grand Island Biological Co., Grand Island, New York, 14072 and Berkeley, California.

Methods.

I. Preparation of L-5178Y Lymphoblast Culture.

Fresh L-5178Y lymphoblast cells were obtained from the peritoneal cavity of male BDF mice which had been transplanted with 1×10^7 tumour cells six to ten days previously. The L-5178Y cells were then washed with Hank's Balanced Salt Solution* twice and re-suspended in Fischer's Medium in spinner flasks at a concentration of 1×10^4 cells per ml. The medium was supplemented with 15 percent horse serum, 100 units per ml. of penicillin, 100 mcg. per ml. of streptomycin and 2.0 mcg per ml. of lyophilized fungizone*. Changes of medium were carried out weekly and whenever necessary.

II. Preparation of Human Embryonic Fibroblast Culture.

Human fetal kidneys were dissected out under sterile condition and homogenized. The homogenate was then inoculated into growth medium. Fetal amniotic fluid was inoculated directly into growth medium and infant skin biopsy was cut into small implants before being placed in growth medium. All human cells were grown in monolayer in RPMI or Minimal Essential Medium, supplemented with 15 percent fetal calf serum* and antibiotics. It usually takes seven to ten days after the

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* Grand Island Biological Associates, Inc.

initial inoculation for the fibroblasts to achieve confluency in a 75 cm^2 Falcon disposable culture flask*. As soon as confluency was obtained, the fibroblasts were trysinized with 0.25 percent trypsin** and re-suspended in a split ratio of 10:1 in Minimal Essential Medium. With a split ratio of 10:1, it would take a theoretical 3.3 doublings for the cells to achieve confluency again (Hayflick and Moorhead, 1961).

III. Inactivation of Sendai Virus.

Inactivation of live Sendai virus was carried out according to the method of Harris (Harris and Watkins, 1965). Appropriate concentration of virus suspended in 1 ml. of Hank's Balanced Salt Solution was placed in a sterile petri dish and irradiated at a distance of 10 cm. with a UV light for three to five minutes***. The infectivity of the irradiated virus was estimated on cultures of monkey kidney fibroblasts through the courtesy of Dr. R. Devine, Director of the Department of Virology, Provincial Laboratory, Edmonton, Alberta. It was found that irradiation up to five minutes reduced the viral infectivity by a factor of $10\text{-}10^2$ and was therefore ineffective. As a result, after the initial experiments with UV irradiated virus, all subsequent experiments were performed with commercial β propriolactone-inactivated Sendai virus.

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* Bio-Cult Canada Ltd., Calgary, Alberta.
** Grand Island Biological Associates Inc. pH adjusted to 7.4.
*** Blak Ray UVL-22, 19 Watts, Ultraviolet Products Inc., San Gabriel, California.

IV. Somatic Cell Hybridization Using Inactive Sendai Virus.

Virus-induced hybridization experiments were performed in the manner described by Okada and Harris (Okada, 1962; Harris and Watkins, 1965). L-5178Y lymphoblasts were gathered from In Vitro culture, spun down at 800 rpm for five minutes and re-suspended in Hank's Salt Solution at a concentration of 5×10^6 cells per ml. Human embryonic fibroblasts during the late phase II growth, that is, after at least 30 doublings, were used because (a) there is evidence that older cells have higher fusion capacity than younger ones, (Okada and Murayama, 1965) and (b) the older fibroblasts would have a limited life span and therefore would be automatically eliminated during subsequent culture (Hayflick, 1965). The fibroblasts were trypsinized with 0.25 percent trypsin, spun down and re-suspended in Hank's Balanced Salt Solution at a concentration of 5×10^6 cells per ml. The pH of the Hank's Salt Solution used in hybridization was adjusted to 7.6 to allow for the acidifying effect of high concentration of cells. Cell counting was done in a hemocytometer and checked with a Coulter Counter. Hemocytometer cell counting was found to be less consistent than the Coulter Counter and yielded results with a variation up to $\pm$ 20 percent. All subsequent cell counting was therefore performed in the Coulter Counter, whenever possible. Half a ml. of each of the cell suspensions were mixed in a pre-chilled, sterile 25 ml. conical flask and 2000 HAU inactive Sendai virus in 1/2 ml. of

Hank's Salt Solution was added to the mixture. The mixture was kept at 4°C for 15 minutes with thorough shaking at five minute intervals and then transferred to a shaker bath at 37°C, running at 100 excursions per minute, for 30 minutes. The cells were then washed with Hank's Solution twice and re-suspended in Fischer's Medium and Minimal Essential Medium or RPMI Medium. The medium was gassed with five percent CO_2. After 48 hours, the post-fusion cells separated into two groups: cells in suspension and cells in monolayer. The suspension cells were poured into new culture flasks and grown separately. Karyotype analysis was carried out at regular intervals.

V. Control Hybridization Experiments.

A control experiment was carried out in exactly the same manner as above except that only L-5178Y cells were used.

VI. Preparation of Chromosome Spreads.

Chromosome analysis was performed according to the methods of Rothfels and Siminovitch, with some modification (Rothfels and Siminovitch, 1967).

(1) For Cells in Suspension - "S" Cells.

Re-suspension in or addition of fresh medium to the cell culture was done 48 hours prior to chromosome preparation. Colcemid

at a concentration of 0.5 mcg. per ml. was added and the cells incubated at 37°C for five hours. Each cell suspension was spun down at 800 rpm for five minutes. The supernatant was discarded, and 10 ml. of 1:5 dilution of Hank's Balanced Salt Solution was slowly added to the cell button. The cells were dispersed gently in the hypotonic solution and left at 37°C for 45 minutes, then spun down and the supernatant discarded. Slowly and without disturbing the packed cells, 10 ml. of freshly prepared glacial acetic acid-methanol fixative* was added. The test tube was refrigerated at 4°C overnight. Following this, the cells were re-suspended in the fixative, spun down, and the fixative discarded. New fixative was added for final re-suspension. A test slide was made by dropping a small amount of cell suspension from a pipette onto a clean slide. If satisfactory chromosome spreads were observed at this point, permanent slides were prepared. If the spreads were unsatisfactory, the fixative was changed several times more. All permanent slides were stained in 2 percent aceto-orcein for 30 minutes and cresyl violet for 45-60 minutes, and cover-slipped for microscopic examination.

(2) For Cells in Monolayer - M Cells.

Monolayer cells for chromosome spreads were grown on glass slides in Leighton culture tubes or directly on Falcon disposable 30 cm^2 culture flasks. Colcemid at 0.5 mcg/ml was added to the monolayer culture, usually 48 to 72 hours after subculture when mitotic

* Acetic acid and methanol in ratio of 1:3.

activity was at its highest point. After five hours of incubation, the medium was decanted and hypotonic salt solution was added. The cells were left at 37°C for one hour. Slowly and gently, fresh fixative was added drop by drop to the hypotonic solution. After five minutes, half of the hypotonic solution was replaced by fresh fixative. After a further five minutes, it was completely replace by fresh fixative and left for a further ten minutes. The slides or flasks were left to air-dry at room temperature and then stained with cresyl violet for 30 minutes. Permanent slides were coverslipped and examined for karyotype.

Results.

I. L-5178Y Lymphoblast Cell Line.

The L-5178Y cells are small round lymphoblasts, measuring 12-16 u in diameter, being highly refractile under the phase contrast microscope. On staining with Wright's stain, the nuclei are basophilic and very large, filling almost the whole of the cytoplasm which forms a very thin peripheral rim (Figure I). The cells grew vigorously In Vitro in spinner culture. The generation time of L-5178Y cells was between 15 and 16 hours under optimal growth condition in Fischer's Medium, supplemented with 15 percent horse serum*. From an initial concentration of 1×10^4 to 5×10^4 cells per ml., approximately 15 to 20 times the numberof viable cells could be expected in 72 hours. In contrast, the lymphoblasts did not grow well in Minimal Essential Medium supplemented with 15 percent calf serum, multiplying initially for 24 to 48 hours before they began to degenerate. (Figure 2). Several attempts to peretuate an In Vitro culture of L-5178Y cells in Minimal Essential Medium failed.

The L-5178Y cells have a bimodal distribution of chromosome numbers. 70 to 90 percent of the cells have a moderately strong mode at 42, the remaining ten to 30 percent of cells are tetraploid or hypotetraploid, close to the double stemline number (2s) of

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* The generation or doubling time $t_g = t/3.3 \times \text{Log}_{10}(N/N_0)$ where T = time in the exponential growth phase; N = number of cells at t; N_0 = number of cells initially.

chromosomes (Figure 3).

The proportion of tetraploidy appears to vary with culture conditions; long-term In Vitro culture is associated with a lower proportion of tetraploid cells than In Vivo propagation.

The Lymphoblast line appeared to have changed its karyotype within the last three years. When it was studied in our laboratory in 1970, it had no marker chromosome (Janzen, 1970). During the past year of culture, however, a marker chromosome was consistently found. (Table I). This is a metacentric chromosome and is also the largest of all the mouse chromosomes which are either acrocentric or telocentric (Figure 4 and 5, Table II). Endoreduplication was often encountered during karyotype analysis of the L-5178Y line (Figure 6).

II. Human Embryonic Fibroblasts.

The human cells have typical elongated, spindle-shaped fibroblastic appearance, and display marked contact inhibition in culture. On staining with Wright's stain, the nuclei are large, oval-shaped pale or eosinophilic with one to four distinct nucleoli. The cytoplasm is abundant and fibrillar at its margin (Plate 1 and 2).

Human fibroblasts grew well in either Minimal Essential Medium or RPMI Medium, both supplemented with 15 percent fetal calf serum. Subculturing with a split-ratio of 10:1, the doubling time of the human fibroblasts was estimated to be approximately 24 hours. The

karyotype is diploid with 46 chromosomes, classified according to the Denver Convention (Figure 7 and 8).

III. Analysis of Mixed Human-Mouse Cell Culture After Viral Treatment.

A. Post-fusion Giant Cells.

Immediately after viral treatment, many giant cells of varying sizes were observed (Figures 9 and 10). Within the first 24 hours, the mixed human and mouse cells separated into two groups: those in suspension, hereafter referred to as "S" cells, and those in monolayer, designated as "M" cells.

B. Karyotype Evolution of "S" Cells.

The "S" cells were morphologically indistinguishable from the L-5178Y lymphoblasts. Initial karyotype analysis showed that the majority of these cells were murine in origin. However, by 72 hours, 20 percent of the "S" cells were found to contain one or more bi-armed chromosomes distinctly different from the murine marker chromosome. The proportion of these presumed hybrid cells rose to nearly 35 percent by ten days, then declined to about ten percent by the end of one month, less than five percent by two months and disappeared altogether by the end of the third month (Figure 11).

The karyotype of the "hybrid cells" appeared to have a bi-modal distribution, with one group of cells clustering around a strong

mode of 40 and the other group having a wider scatter around 80. The latter group declined faster during culture and disappeared by the end of the first month; it persisted slightly longer in RPMI Medium (Figures 12 and 13). Both groups of hybrid cells contained only a few number of human-like bi-armed chromosomes; the majority had only one such chromosome instead of or in addition to the murine marker chromosome. The maximum number of human-like bi-armed chromosomes contained in the hybrid cells was five.

Hybrid cells which contained around 80 chromosomes were either hypo or hypertetraploid in relation to the L-5178Y lymphoblasts; they contained, in addition to the human-like bi-armed chromosomes, approximately the 2s number of murine telocentric and acrocentric chromosomes.

(ii) Analysis of the Pattern of Human Chromosomes Retained in Hybrid "S" Cells.

Morphological distinction of human chromosomes from the murine karyotype is not difficult, but not completely foolproof. Human Group A,B, E and F chromosomes are distinct from the murine counterpart; Group D and G human chromosomes, on the other hand,could be confused with the murine acrocentrics, and the distinction, though possible, cannot be absolutely certain. In addition the mouse marker submetacentric chromosome could be mistaken for some of the human C

chromosome, and less likely, the number 2 human submetacentric. With this in mind, the pattern of human chromosomes retained in the hybrid "S" cells was analysed by two or more independent observers (Figures 14 and 15). It can be seen from the histograms that the observed pattern of human chromosomes in the hybrid "S" cells was independent of the culture medium. The most striking feature was that human chromosomes of the "A" group seemed to be preferentially retained. Approximately 60 percent of the hybrid "S" cells analysed over a period of four months contained one or more group "A" human chromosomes. By contrast, only 12 percent of the hybrid "S" cells retained human chromosomes of the "B" group, the next most frequently retained group (Table III).

The commonest hybrid "S" cells contained around 40 to 42 chromosomes, having a large metacentric chromosome presumably derived from the human parent in addition to the submetacentric murine chromosome (Figures 16,17,18,19). The miscellaneous group of chromosomes in these hybrid cells (Figures 14 & 15), consisted of extra-long telocentric or submetacentric chromosomes and ring chromosomes which resembled neither of the parental chromosomes (Table IV).

(iii) Control Experiments with L-5178Y Lymphoblasts.

The large human-like metacentric chromosome observed in the

majority of hybrid "S" cells could have arisen from misdivision of centromere or centric fusion of two murine telocentric chromosomes under the influence of the Sendai Virus. To exclude this possibility control experiments were performed in the exact manner as in the human-mouse hybridization experiments except that only L-5178Y lymphoblasts were used. Karyotype analysis was carried out at regular intervals over one month.

None of the cells analyzed in this group of experiments contained a human "group "A" like" chromosome. There was, however, a tendency towards a greater proportion of hypertetraploidy and hypotetraploidy as compared with a controlled L-5178Y lymphoblast culture. A small proportion of these cells, about five percent, contained more than the double stem line (2S) number of murine chromosomes. Occasionally one or two of these cells were found to contain nearly 200 murine chromosomes (Figures 20 and 21).

C. Karyotype Evolution of "M" Cells.

The post-fusion monolayer cells were of two distinct morphological types. The majority resembled the human fibroblasts. The rest were small round cells, morphologically indistinguishable from the murine parent, scattered in small clusters amongst the fibroblasts. Although the round cells adhered to the culture flask, they

were easily detachable by simple agitation. In contrast, the fibroblast-like "M" cells adhered firmly to the glass surface and were freed only after incubation with 0.25 percent trypsin at 37°C for 15 - 30 minutes. A five minute treatment with trypsin cleared the culture flask almost completely of the round cells, leaving behind the fibroblastic "M" cells. Karyotype analysis of the detached round cells revealed their murine origin. This indicates that the L-5178Y cells were able to attach to the culture flask by virtue of the fibroblast monolayer. By a simple experiment wherein L-5178Y lymphoblasts were added to a fibroblast culture this impression was confirmed. Within minutes, small clusters of L-5178Y cells attached themselves to the culture flask amongst the monolayer fibroblasts.

Using the principle outlined above, the post-fusion monolayer culture was repeatedly agitated and treated with trypsin until a pure culture of fibroblastic "M" cells was obtained. The latter was observed under the phase contrast microscope and karyotyped at regular intervals. During the first month of culture, many bizaarre shaped heterokaryons were observed. They contained one or more large oval, pink staining nuclei of human fibroblast origin, and anything from one - 20 murine nuclei which were smaller, round-shaped and stained dark purple with Wright's stain (Plate III, IV).

Initially, weekly karyotype studies of the "M" cells failed

to reveal any hybrid cells. All the cells analysed during the first month were human in origin.

At the end of the second month of culture a new hybrid cell type emerged. These hybrid cells contained the full chromosome complement of the murine parent and from 10 to 20 distinct bi-armed chromosomes attributable to the human karyotype (Figures 22 and 23).

Unfortunately these hybrid "M" cells occurred very infrequently totalling less than one percent of the "M" cell culture.

DISCUSSION

I. Interpretation of Results.

A. Hybrid Cells.

Two distinct hybrid populations have been produced in these experiments. The hybrid "S" cells were morphologically indistinguishable from the L-5178Y lymphoblasts; they emerged and increased in proportion within the first month of mixed culture. In contrast, the fibroblast-like hybrid "M" cells became observable only after the first month of culture, and occurred very infrequently, showing no tendency to increase with time.

The reason behind this distinction is not clear. However, both types of hybrid cells did not show a growth advantage over the parental cells and were eventually overgrown.

Morphologically the hybrid "S" cells resembled the L-5178Y lymphoblasts. Both multiplied in suspension culture in Fischer's Medium. On the other hand, the few observed hybrid "M" cells were fibroblastic and grown in monolayer. Their fibroblastic shape and ability to adhere to the culture flask could be attributed to the relatively large number of human chromosomes retained in these hybrids. The human group "A" chromosomes presumably did not confer these properties to the hybrid "S" cells. In these hybrids the murine

karyotype dominates in phenotypic expression.

The morphological and karyotypic characteristics of most of the human X mouse somatic cell hybrids described in the literature resembled those of the hybrid "M" cells in our experiments, being fibroblasts and growing in monolayer culture. Few authors have produced two distinct hybrid populations from the fusion of a mouse cell line with a human cell line or strain, as in our human and mouse combination. This discrepancy could be explained in two ways. Firstly, in most of the hybridization experiments, human and mouse fibroblasts were exclusively used. Secondly, in the few cases where a fibroblast cell line was fused with a suspension cell population, a selective system was usually employed and the suspension cells were discarded during post-fusion changes of medium. Thus Miggiano et al developed a system of human-mouse hybridization in which they hybridized enzyme-deficient mouse L-cell lines with human peripheral white blood cells, taking advantage of the latter's inability to proliferate. In their series of experiments, the suspension cells were discarded 24 hours after fusion and therefore were never subjected to serial karyotype studies. (Miggiano et al, Wistar Institute Symposium). This interpretation is supported by the findings of Koyama et al in their interspecific hybridization experiments. These authors produced 11 hybrid clones between an 8-azaguinine-resistant mouse cell line (FC-1), which was a floating cell line, resembling in morphology the L-5178Y lymphoblasts, and a fibroblastic Chinese hamster lung cell line (CHL).

(Koyama et al, 1970). It was found that morphologically, the hybrid clones fell into three types: (1) fibroblastic and adherent, designated "A" type, (2) floating and round, the "F" type, and (3) mixture of both, the "M" cell type. By correlating the distinct morphology of each hybrid clone with the karyotype, Koyama et al were able to attribute the fibroblastic morphology of the hybrid clones to the presence of hamster bi-armed chromosomes.

B. Selective Retention of Group "A" Human Chromosomes in Hybrid "S" Cells.

Rapid and marked segregation of human chromosomes is a feature of human and mouse somatic cell hybrids. The hybrid "S" cells produced in these experiments exhibited a strikingly early and extensive loss of human chromosomes. Most of these hybrid cells contained only one or two human chromosomes. Analysis of the pattern of human chromosomes observed in these hybrid "S" cells demonstrated that in this particular combination, segregation of human chromosomes did not occur at random though no selective methods were employed. Human Group "A" chromosomes were selectively retained in these hybrid cells. 60 percent of the hybrid "S" cells contained one or more human "A" group chromosomes. By contrast, only 12 percent of the hybrid cells retained human chromosomes of the "B" group, the next most frequently retained group. This finding differs from previous experiments by other authors.

In all of the human mouse somatic cell hybrids so far produced, the distribution of human chromosomes in the hybrid cells occurred at random except in cases where a selective procedure was used. For instance, when a mouse cell line lacking thymidine kinase was hybridized with a human cell line or strain and culture in Littlefield's Selective Medium, selection favoured the retention of the human chromosome containing the gene for thymidine kinase. It was in this way that the gene for thymidine kinase activity was assigned to a human "E" group chromosome. Many other enzyme activities have been linked similarly to a particular chromosome. In cases where no selective procedures were employed, Marin and Nabholz et al, have provided indirect statistical evidence that in some instances of interspecific hybrids, segregation of chromosomes from one parent might have occurred in "orientated groups". Our experiments showed that non-random segregation of human chromosomes can occur in a human-mouse somatic cell hybrid.

The factors favouring human group "A" chromosome retention in the hybrid "S" cells are not known. There may be an association or compatibility between human "A" group chromosomes and the L-5178Y lymphoblasts, or it may be that the human "A" chromosomes contribute some factor(s) to the mouse cells, which are beneficial to their growth in suitable culture media. The fact that the human group "A" chromosomes bear physical resemblance to the murine marker may be important in this regard. In any event, the human chromosomes do not seem to enhance the <u>In Vitro</u> growth potential of the mouse lymphoblasts, as judged from the declining proportion of the hybrid "S" cells in

In Vitro culture. It is not known whether the human "A" chromosomes are wholly or only partially active in the hybrid state. Nor is it known if the hybrid "S" cells, when implanted In Vivo in the murine hosts, are more or less malignant than the parent lymphoblasts. Thus, it follows that the next step would be to isolate a pure culture of hybrid "S" cells in order to be able to find answers to many important questions that arise from these experiments.

II. Criticism of Methods.

The karyotype distinction between the L-5178Y lymphoma cells and the human fibroblasts is such that identification of the hybrid cells by chromosome analysis is not difficult. Careful analysis of each photograph of a hybrid cell by two or more independent observers reduces observer bias to a minimum. The murine karyotype consists of acrocentric and telocentric chromosomes with only one submetacentric marker per cell. Thus, the presence of a number of bi-armed chromosomes, especially of varying lengths, in a predominantly acrocentric and telocentric karyotype identifies the cell as a hybrid. In this regard the hybrid "M" cells are undoubtedly genuine hybrids. However, where only one or two human-like bi-armed chromosomes are present in a predominantly murine karyotype, the question arises whether these are in fact human in origin or murine resulting from mis-division of centromere or centric fusion of two telocentric chromosomes. The latter reactions may presumably occur under the influence of Sendai virus. This possibility is rendered unlikely by repeated control

experiments in which L-5178Y lymphoblasts alone were treated with Sendai virus in exactly the same experimental condition as the interspecific hybridization experiments. Repeated chromosome analysis failed to show any human-like chromosomes in these cells over a one month period. No large metacentric chromosomes resembling those of the human "A" group were observed in these control experiments. The Author believes that the commonly seen "S" cells, which contain one or more large metacentric human group "A"-like chromosomes in addition to a complete set of murine karyotype are genuine hybrid cells. None the less, absolute identification of the human chromosomes in hybrid "S" cells can only be achieved if one can isolate a pure hybrid culture. The latter can then be subjected to chromosome analysis and differential staining by any one of the existing techniques such as; fluorescence banding with guinacrine mustard, giemsa staining, or C banding (Caspersson et al, 1970; Harris et al, 1971c; Sumner et al, 1971).

The hybrid "M" cells emerged late in the culture and in such small numbers that the analysis of the karyotypic evolution and the pattern of human chromosomes retention was not possible. Two techniques were employed to increase the proportion of hybrid "M" cells in our laboratory: 1. Ephrussi et al observed their interspecific hybrids, which were overgrown by the parental cells in continued culture at 37°C, became dominant at 29°C and finally

eliminated the parental cells altogether. Using the same principle, the Author incubated the monolayer "M" cells at 29°C for a prolonged period (three weeks). The proportion of hybrid cells was observed to increase to around one percent. Unfortunately, this culture was subsequently lost through contamination. 2. In the last couple of experiments human fibroblasts after the sixteenth passage (that is more than 50 doublings) were used in the fusion reaction. It was hoped that the human fibroblasts would, because of their limited life span, disappear spontaneously from the monolayer "M" cells during subsequent culture, thus leaving behind the hybrid "M" cells as a pure culture. This line of research is being followed in our laboratory.

III. Future Studies.

The next step in this research project will be to isolate the hybrid cells. Once isolated, one can study the following:

- the morphology of the hybrid cells and correlation with the hybrid karyotype.
- the evolution and stability of the hybrid karyotype; the pattern of human chromosome retention in the hybrid cells and its evolution, using differential chromosome banding technique.
- the growth characteristics of the hybrid cells both *In Vitro* and *In Vivo*.
- the effect of normal human genetic material on tumour cell behaviour.
- the correlation of cellular behaviour or enzyme activities with the presence of a specific human chromosome in the hybrid cell. For example, the human group "A" chromosomes in the hybrid "S" cells.

Summary and Conclusion.

Human diploid fibroblasts were fused with L-5178Y lymphoblasts *In Vitro* aided by inactivated Sendai Virus. Human-mouse hybrid cells were produced. Karyotype analysis at regular intervals has permitted the following conclusions to be drawn.

1. Two morphologically distinct hybrid populations were produced. One resembled the murine parent, the hybrid "S" cells, and the other the fibroblastic "M" cells.

2. Detailed chromosome studies of the hybrid "S" cells demonstrated non-random segregation of human chromosomes in this particular human-mouse somatic cell hybrid. Human chromosomes of the "A" group were preferentially retained.

3. Presence of human group "A" chromosomes (in the murine karyotype) is not associated with a fibroblastic morphology or enhanced growth rate *In Vitro*.

The research reported in this thesis forms a foundation for future human-mouse hybridization studies which, if successful, will add information of the influence of normal human genetic material on tumour cell behaviour both *In Vitro* and *In Vivo*.

TABLE I.

DISTRIBUTION OF THE MARKER CHROMOSOME IN 100 L-5178Y LYMPHOMA CELLS.

No. of Submetacentric marker chromosome in each cell.	0	1	2
Number of 1s cells	4	77	4
Number of 2s cells	-	-	15

TABLE II.

ARM RATIO OF THE SUBMETACENTRIC MARKER CHROMOSOME AS MEASURED IN 44 PHOTOGRAPHS.

ARM RATIO	NUMBER OF CELLS.
1.5:1	26
1.75:1	14
2:1	4
	44

TABLE III.

PATTERN OF HUMAN CHROMOSOMES RETAINED IN HYBRID "S" CELLS.

	HUMAN CHROMOSOMES	
	GROUP A	GROUP B
No. of hybrid "S" Cells	35 (60) *	7 (60) *
Percentage	60%	12%

* total number of hybrid "S" cells analyzed.

d = 4.32 $p < 0.001$

TABLE IV.

UNUSUAL CHROMOSOMES IN HYBRID S CELLS, NOT PRESENT IN EITHER OF THE PARENT KARYOTYPES

DESCRIPTION OF CHROMOSOME	NUMBER OF CELLS
Extra long telocentric	3
Extra long submetacentric	1
Ring	1
Dot	1

Figure I. L-5178Y Lymphoblasts in hemocytometer.

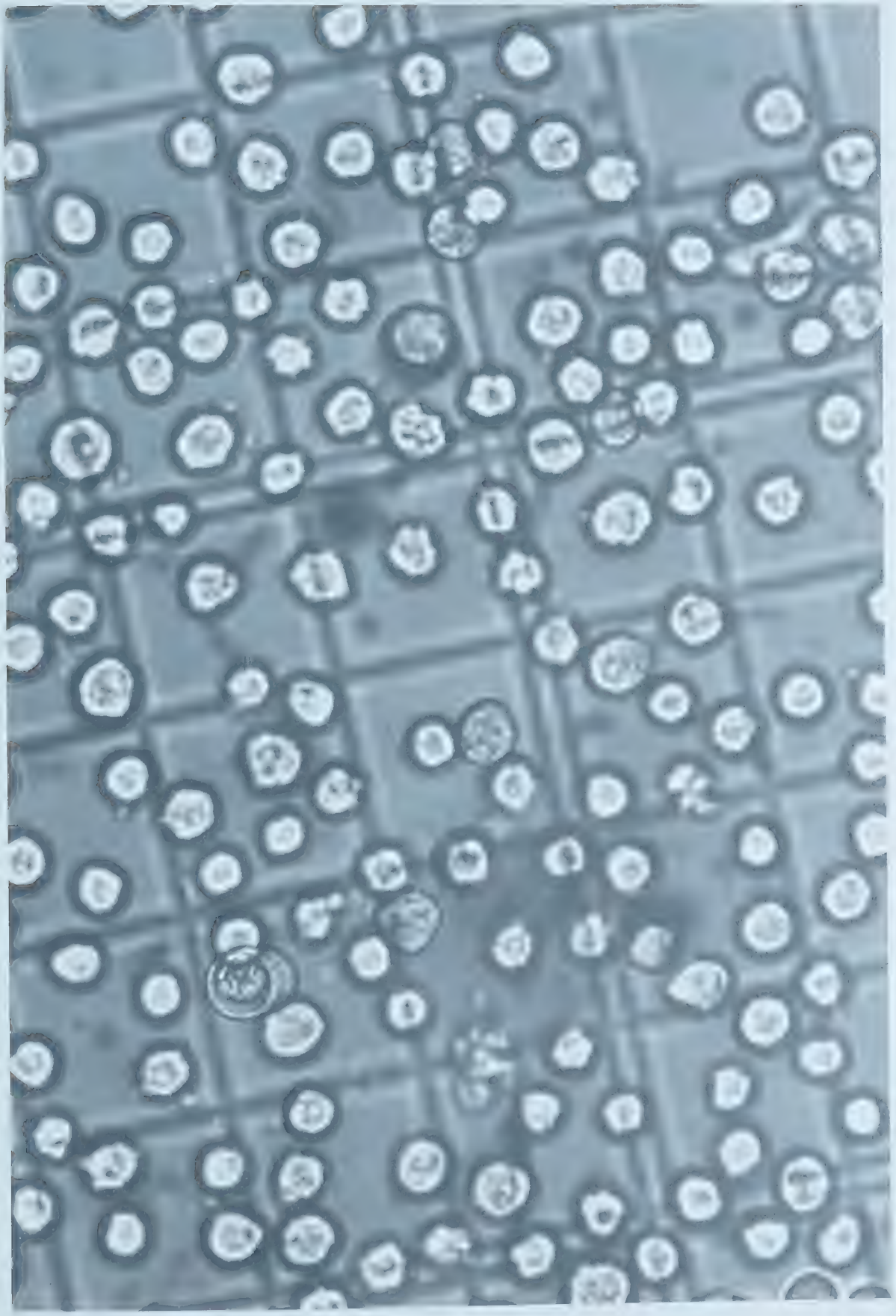


Figure 2. Growth curves of L-5178Y Lymphoblasts in (a) Fischer's Medium and (b) Minimal Essential Medium.

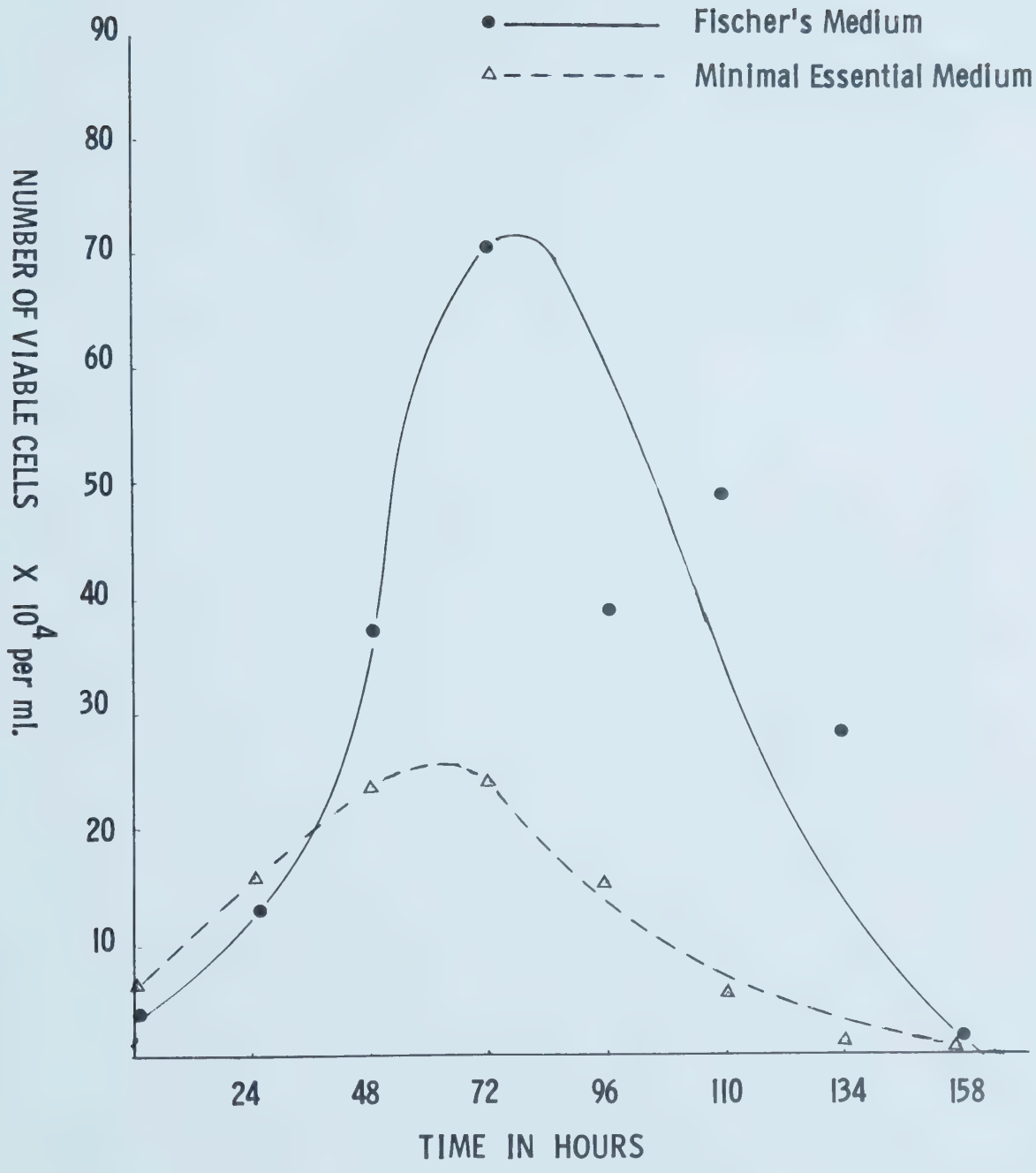

Fischer's Medium
Minimal Essential Medium
NUMBER OF VIABLE CELLS X 10^4 per ml.
90
80
70
60
50
40
30
20
10
24
48
72
96
110
134
158
TIME IN HOURS

Figure 3. Histogram showing the chromosome counts in 100 cells of the L-5178Y mouse lymphoblast line.

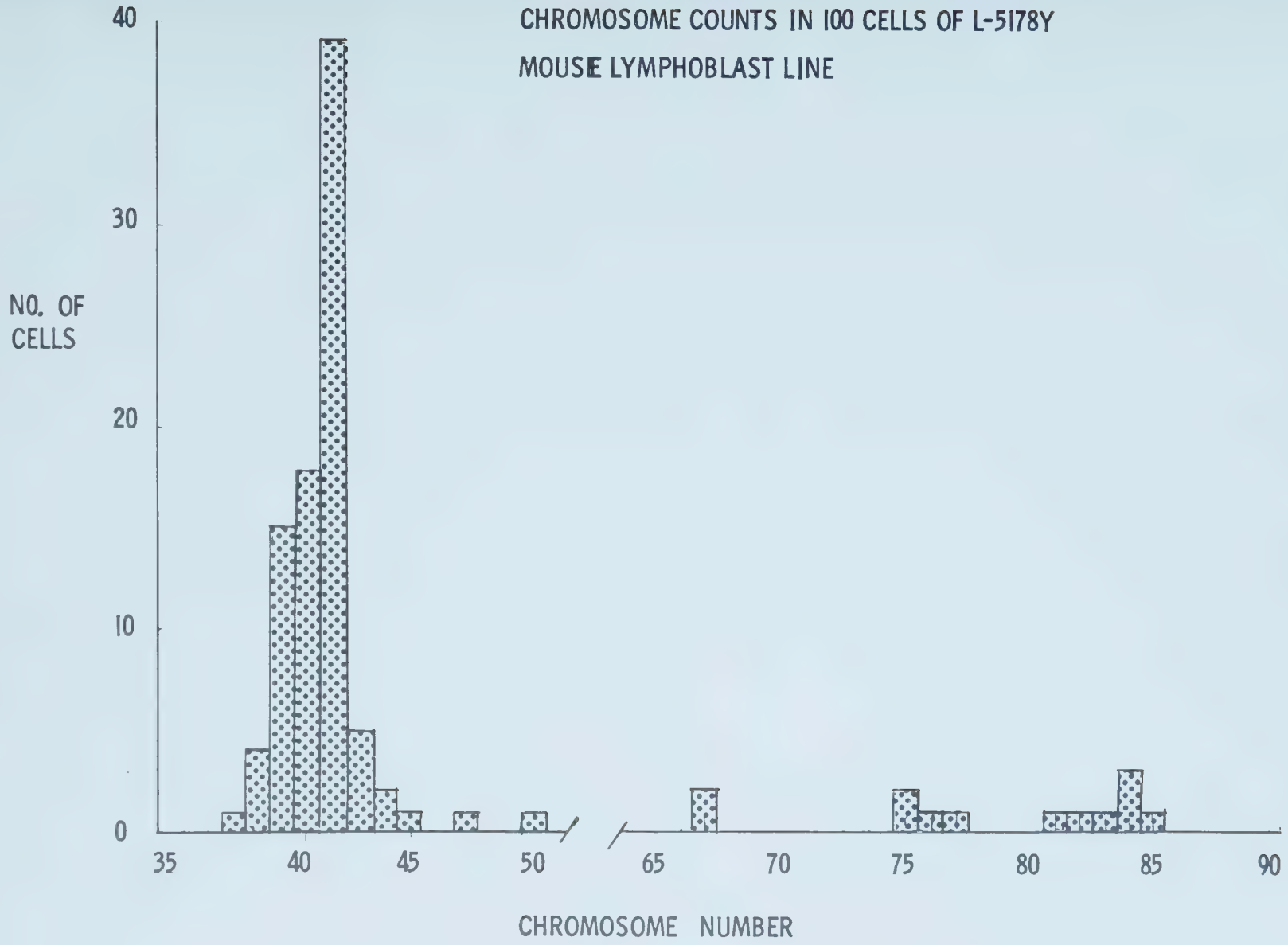

CHROMOSOME COUNTS IN 100 CELLS OF L-5178Y
MOUSE LYMPHOBLAST LINE
NO. OF CELLS
40
30
20
10
0
35
40
45
50
65
70
75
80
85
90
CHROMOSOME NUMBER

Figure 4. Representative Karyotype of the L-5178Y lymphoblast line. (1s cell, 42 chromosomes).

L5178Y LYMPHOBLAST KARYOTYPE

Figure 5. Representative karyotype of the L-5178Y lymphoblast cell line. (2s cell, 84 chromosomes).

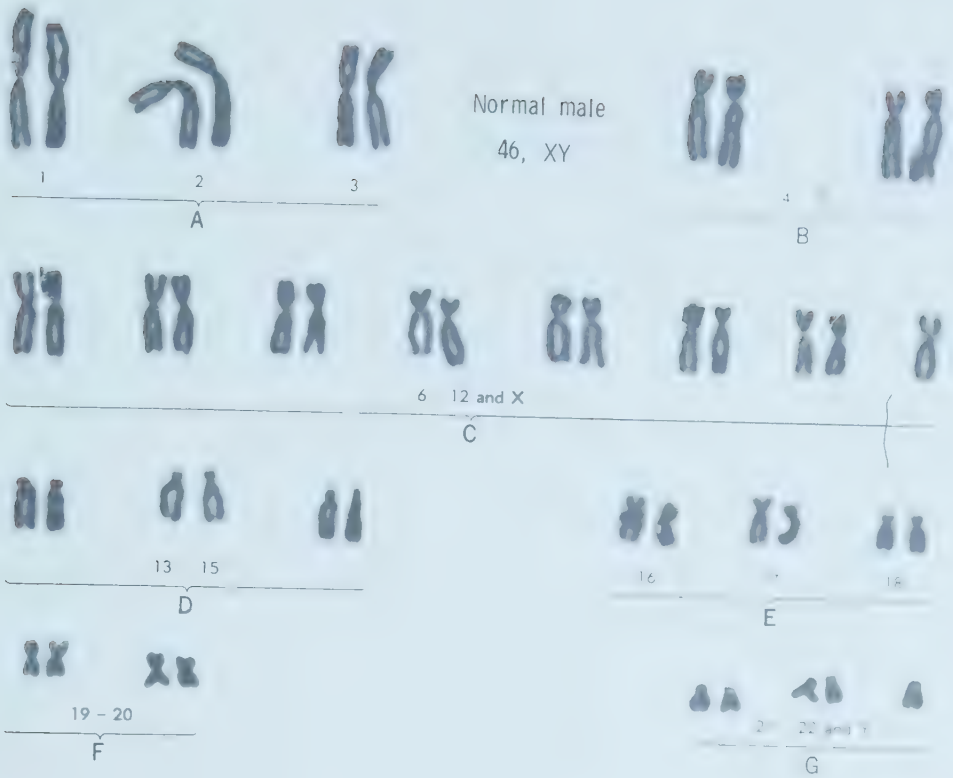
E9 – 25, 26, 27

Figure 6. Endoreduplication of a L-5178Y lymphoma cell.

E4-15

Figure 7. Normal diploid human karyotype, male. (arranged according to the Denver Convention).

Figure 8. Normal diploid human karyotype, female. (arranged according to the Denver Convention).


Normal male
46, XY
1
2
3
A
4
B
6
12 and X
C
13
15
D
16
E
19 - 20
F
G

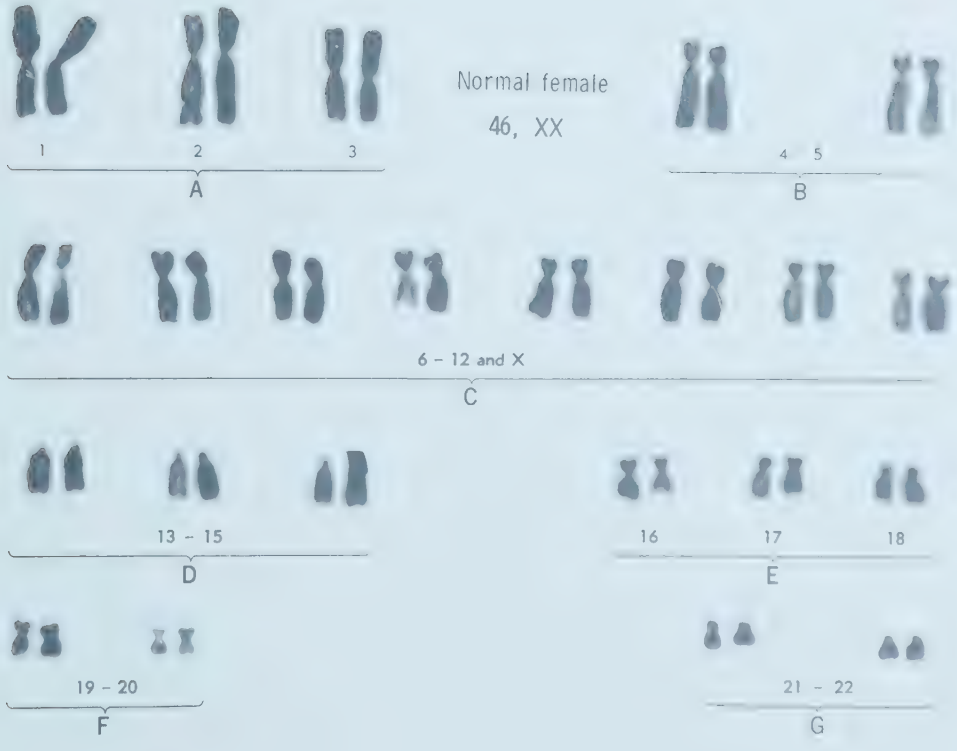

Normal female
46, XX
1
2
3
A
4
5
B
6 - 12 and X
C
13 - 15
D
16
17
18
E
19 - 20
F
21 - 22
G

Figure 9. Post-fusion giant cells occurring immediately after viral treatment of L-5178Y lymphoblasts and human fibroblasts.

Figure 10. Post-fusion giant cells occurring immediately after viral treatment of L-5178Y lymphoblasts and human fibroblasts.

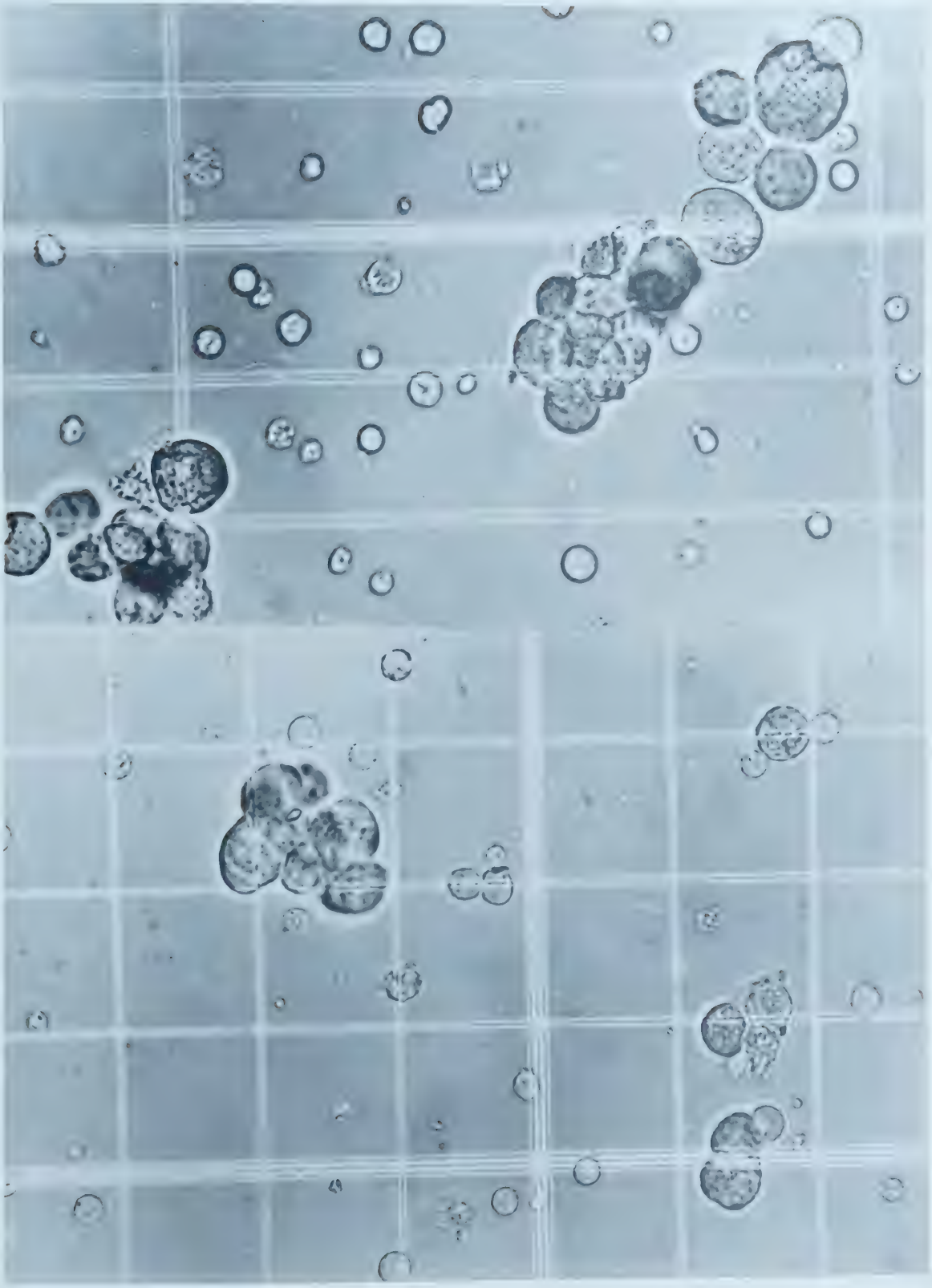


Figure 11. Proportion of hybrid "S" cells in mixed human and mouse cell culture.

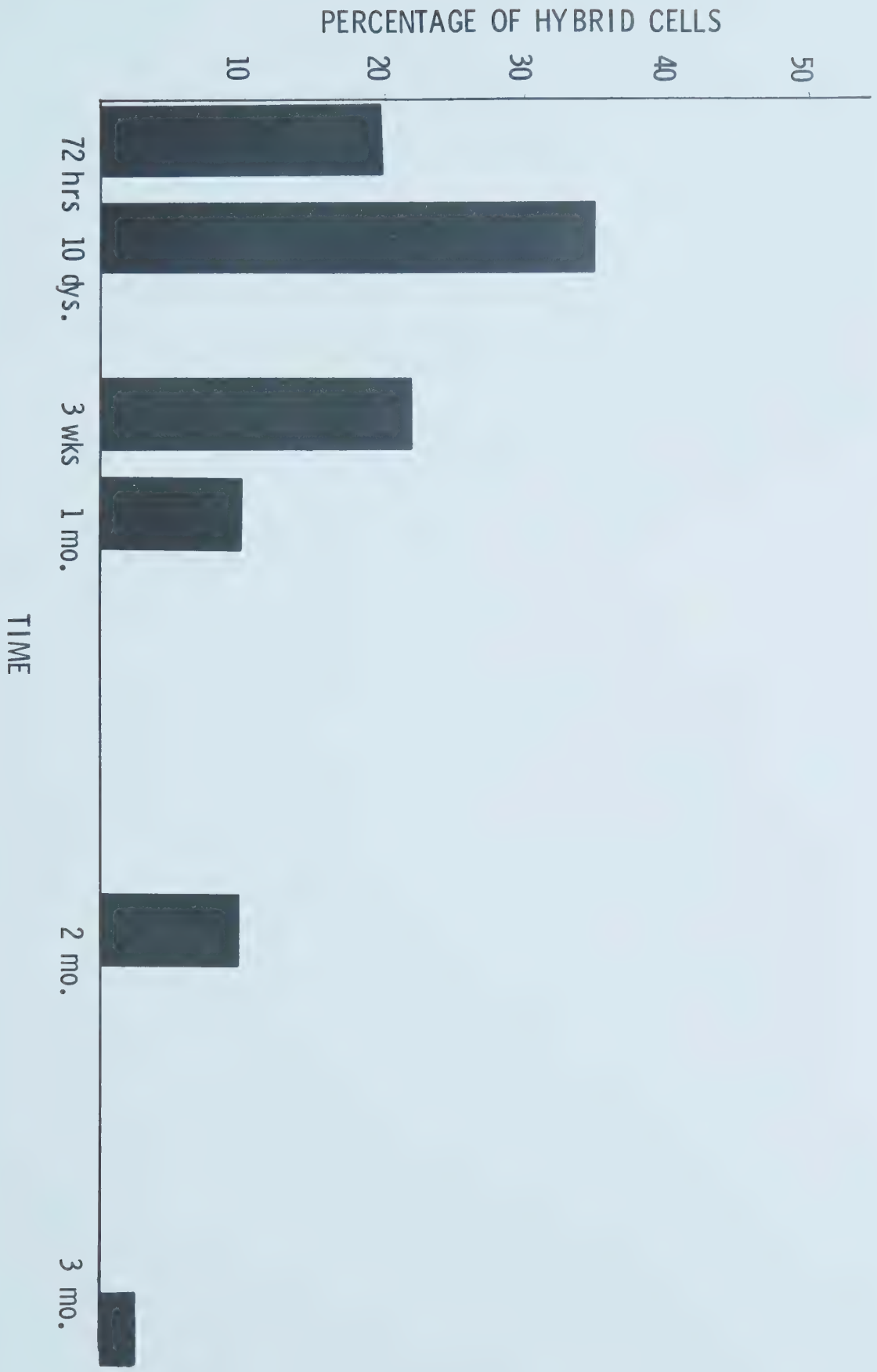
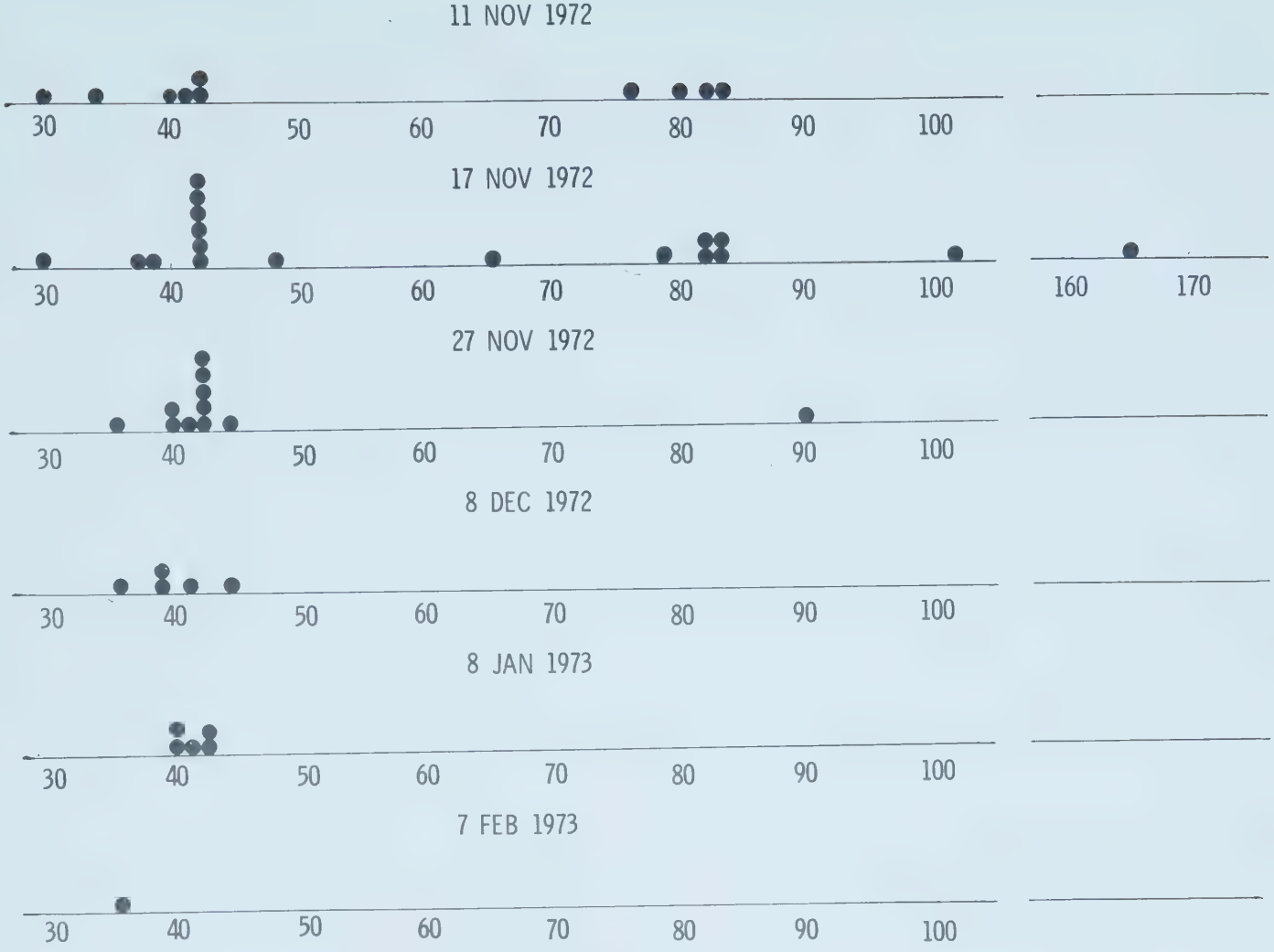

PERCENTAGE OF HYBRID CELLS
10
20
30
40
50
72 hrs
10 dys.
3 wks
1 mo.
2 mo.
3 mo.
TIME

Figure 12. Karyotype evolution of hybrid "S" cells in Fischer's medium. Each dot represents one hybrid "S" cell.

FISCHER'S MEDIUM



NO. OF CHROMOSOMES IN HYBRID CELLS

Figure 13. Karyotype evolution of hybrid "S" cell in RPMI Medium. each dot represents one hybrid "S" cell.

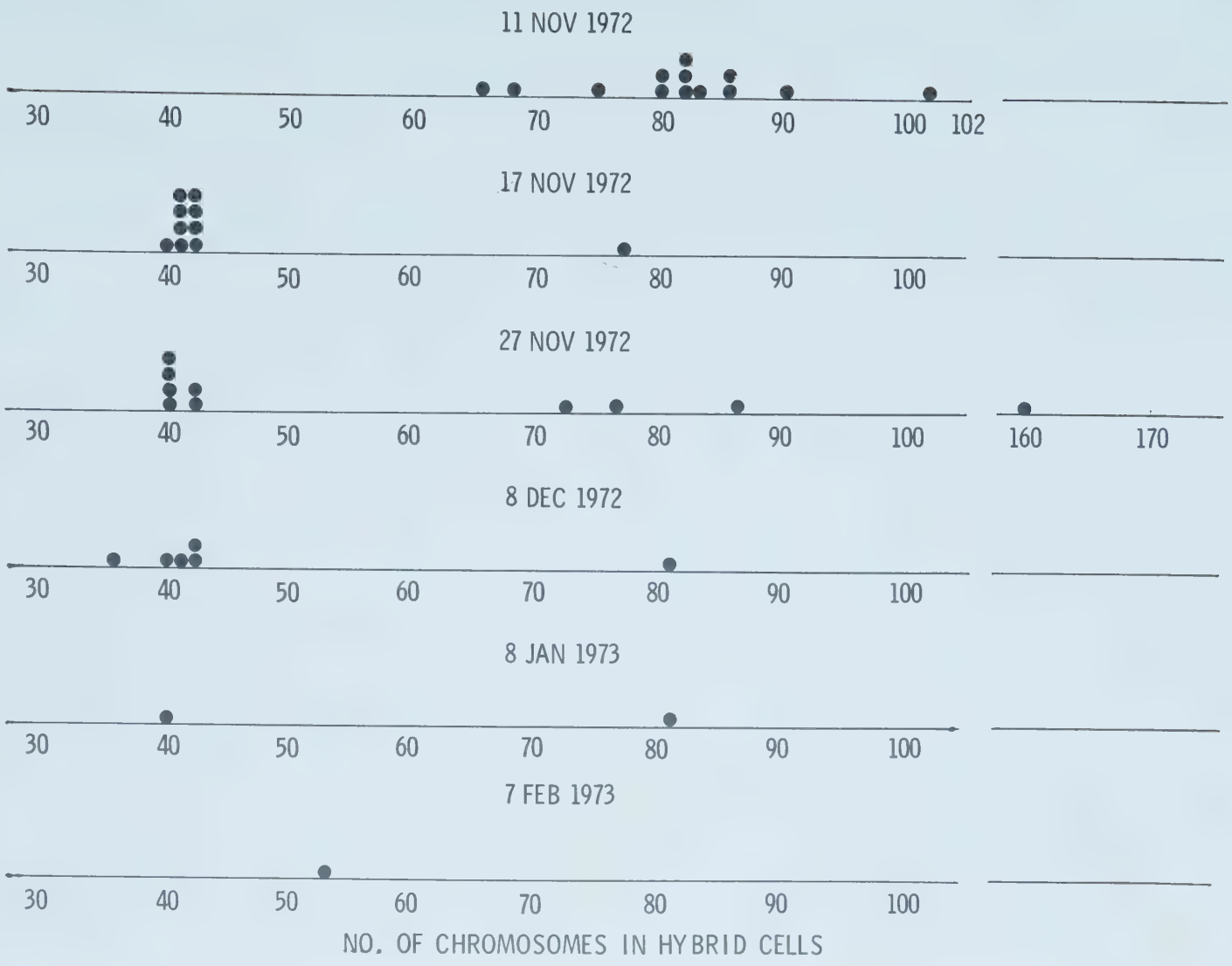
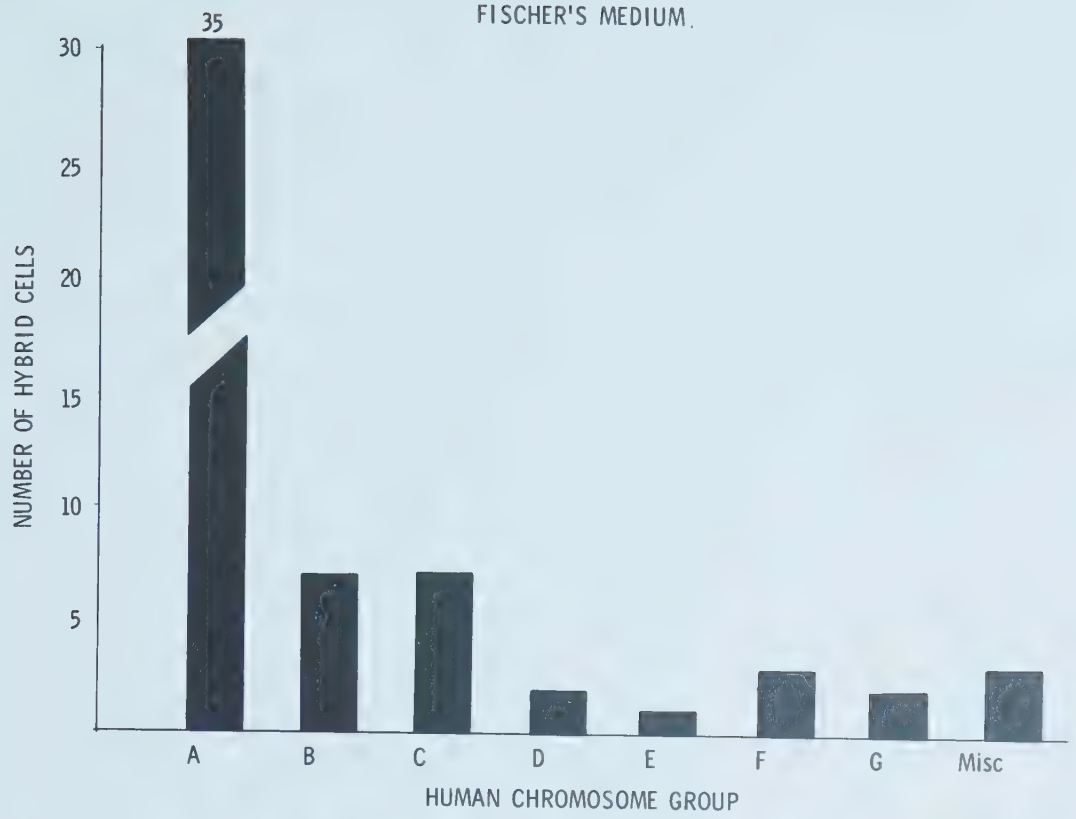

RPMI MEDIUM
11 NOV 1972
30 40 50 60 70 80 90 100 102
17 NOV 1972
30 40 50 60 70 80 90 100
27 NOV 1972
30 40 50 60 70 80 90 100 160 170
8 DEC 1972
30 40 50 60 70 80 90 100
8 JAN 1973
30 40 50 60 70 80 90 100
7 FEB 1973
30 40 50 60 70 80 90 100
NO. OF CHROMOSOMES IN HYBRID CELLS

Figure 14. Histogram illustrating the pattern of human chromosomes retained in hybrid "S" cells, cultured in Fischer's Medium.

Figure 15. Histogram illustrating the pattern of human chromosomes retained in hybrid "S" cells, cultured in RPMI Medium.


FISCHER'S MEDIUM.
35
30
25
20
15
10
5
NUMBER OF HYBRID CELLS
A
B
C
D
E
F
G
Misc
HUMAN CHROMOSOME GROUP

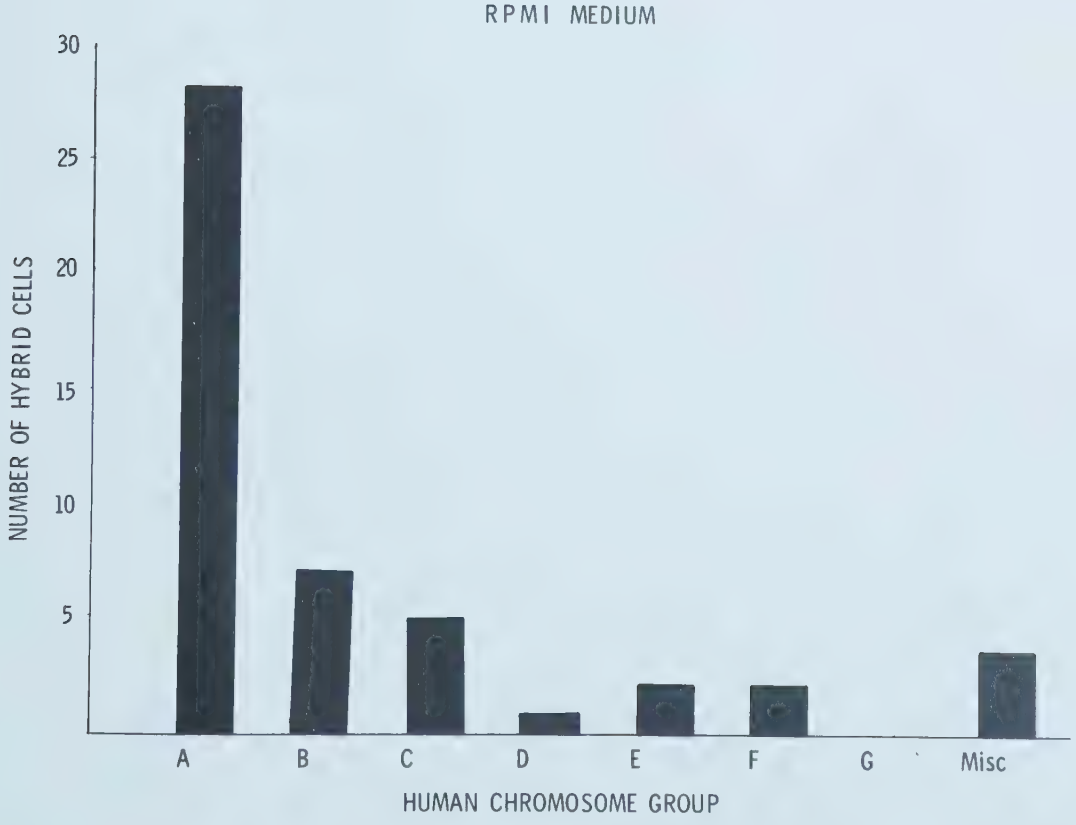
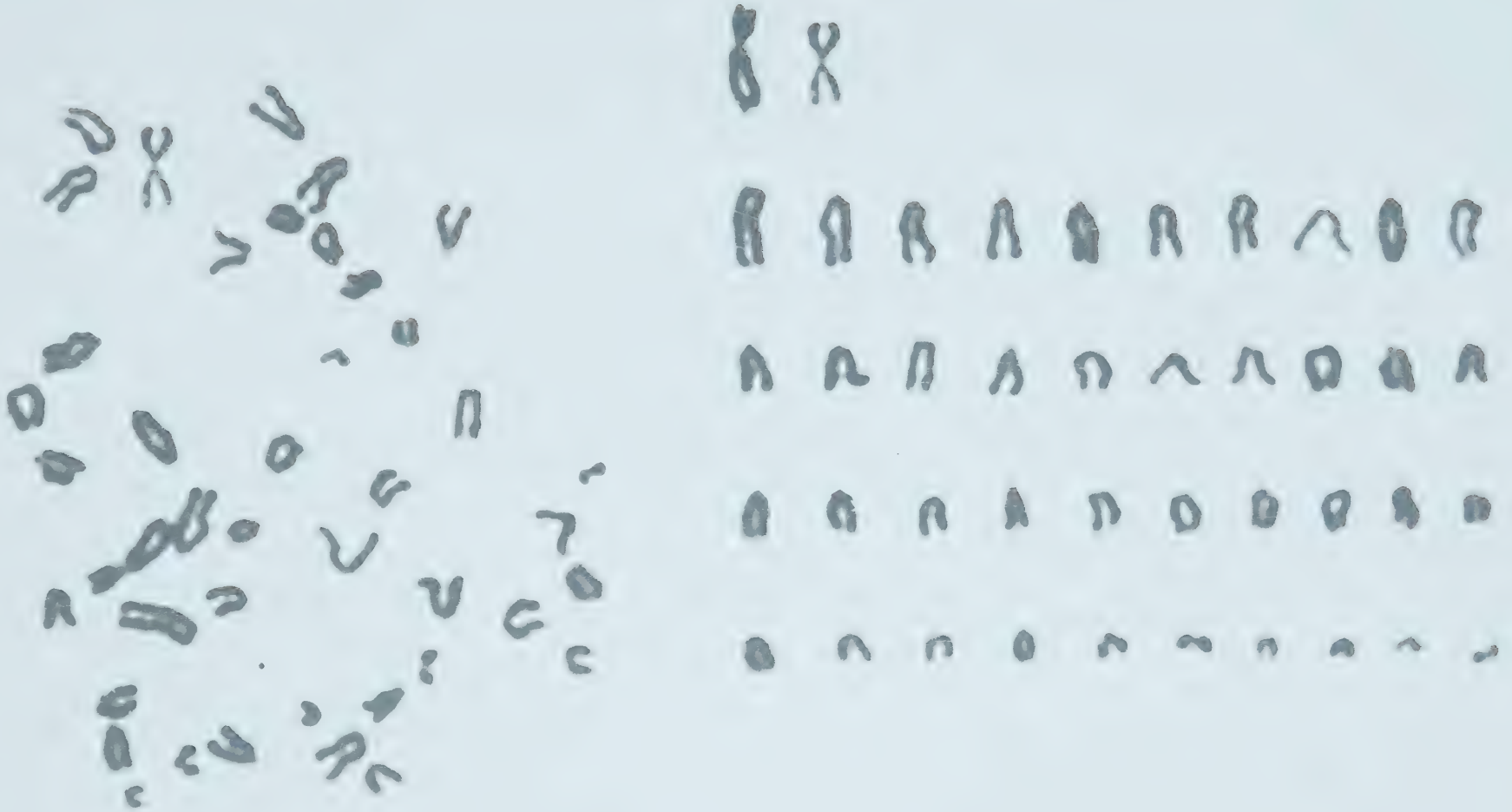

RPMI MEDIUM
30
25
20
15
10
5
NUMBER OF HYBRID CELLS
A
B
C
D
E
F
G
Misc
HUMAN CHROMOSOME GROUP

Figure 16. Typical hybrid "S" cell with the submetacentric mouse marked chromosome and a human "A" chromosome.

HYBRID CELL E8 - 13 FM 17/11/72

Figure 17. Hybrid "S" cell with a human group "C" chromosome.



HYBRID CELL E4-8 RPMI 17/11/72

Figure 18. Hybrid "S" cell with 2 human group "A" chromosomes.

HYBRID CELL E6 - 5,6 FM 17/11/72

Figure 19. Hybrid "S" cell with 5 bi-armed chromosomes.

HYBRID CELL E1 - 21 RPMI 11/11/72

Figure 20. Histogram showing the chromosome counts in fifty L-5178Y cells, two weeks after viral treatment.

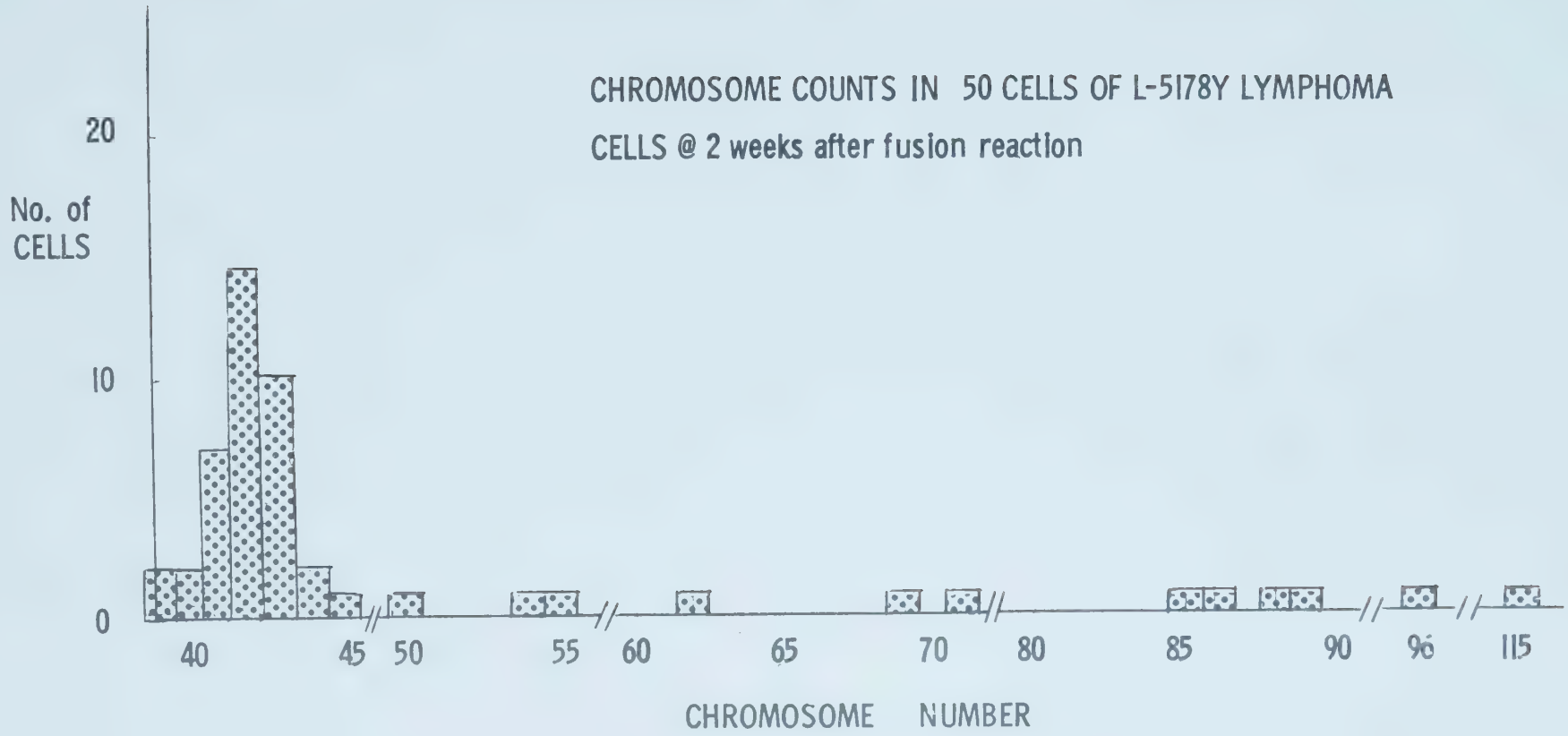

CHROMOSOME COUNTS IN 50 CELLS OF L-5178Y LYMPHOMA
CELLS @ 2 weeks after fusion reaction
No. of CELLS
20
10
0
40
45
50
55
60
65
70
80
85
90
96
115
CHROMOSOME NUMBER

Figure 21. Histogram showing the chromosome counts in fifty L-5178Y cells, one month after viral treatment.

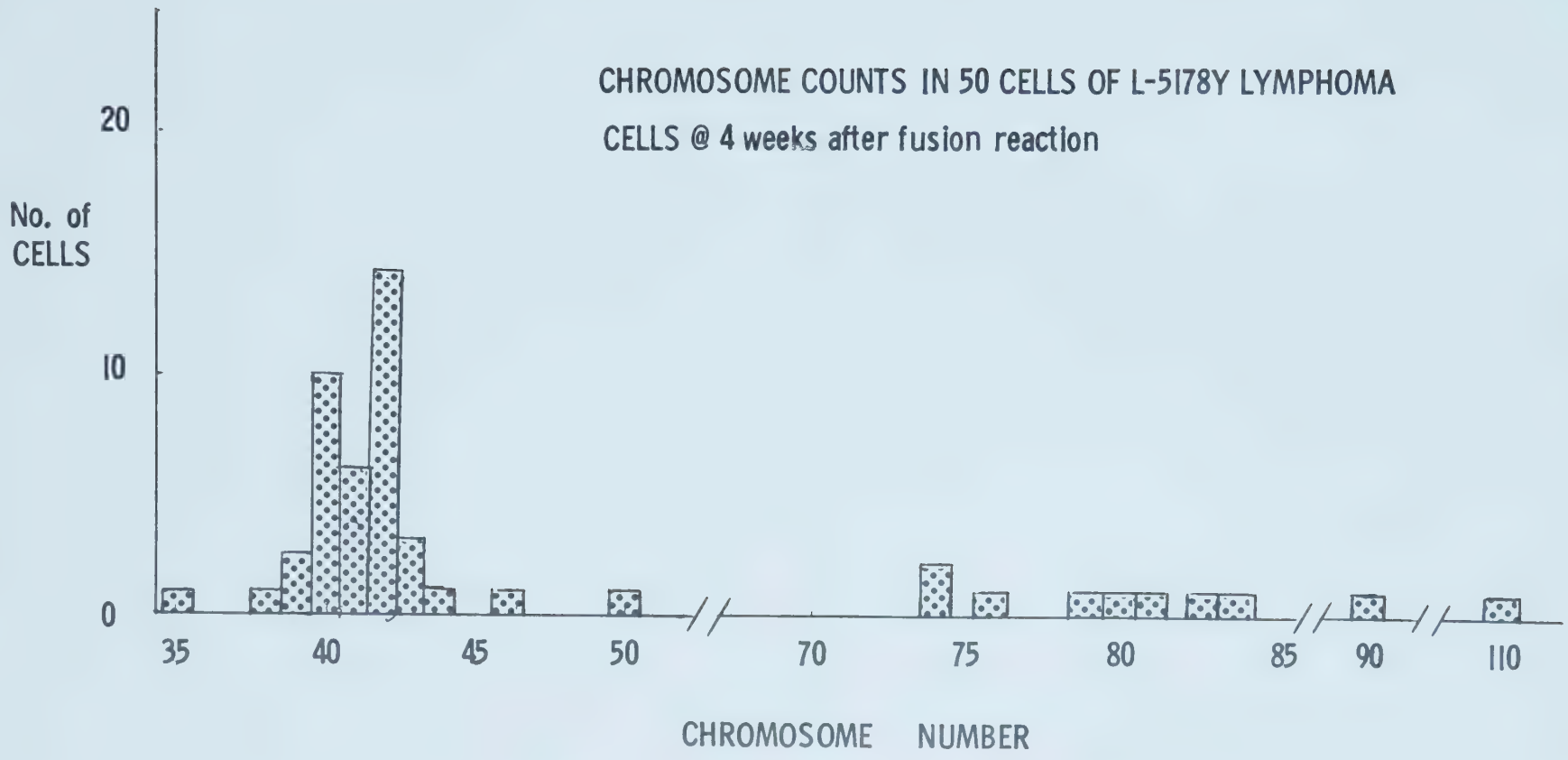

CHROMOSOME COUNTS IN 50 CELLS OF L-5178Y LYMPHOMA
CELLS @ 4 weeks after fusion reaction
No. of CELLS
20
10
0
35
40
45
50
70
75
80
85
90
110
CHROMOSOME NUMBER

Figure 22. Hybrid "M" cell containing approximately 10 - 15 human-like chromosomes.

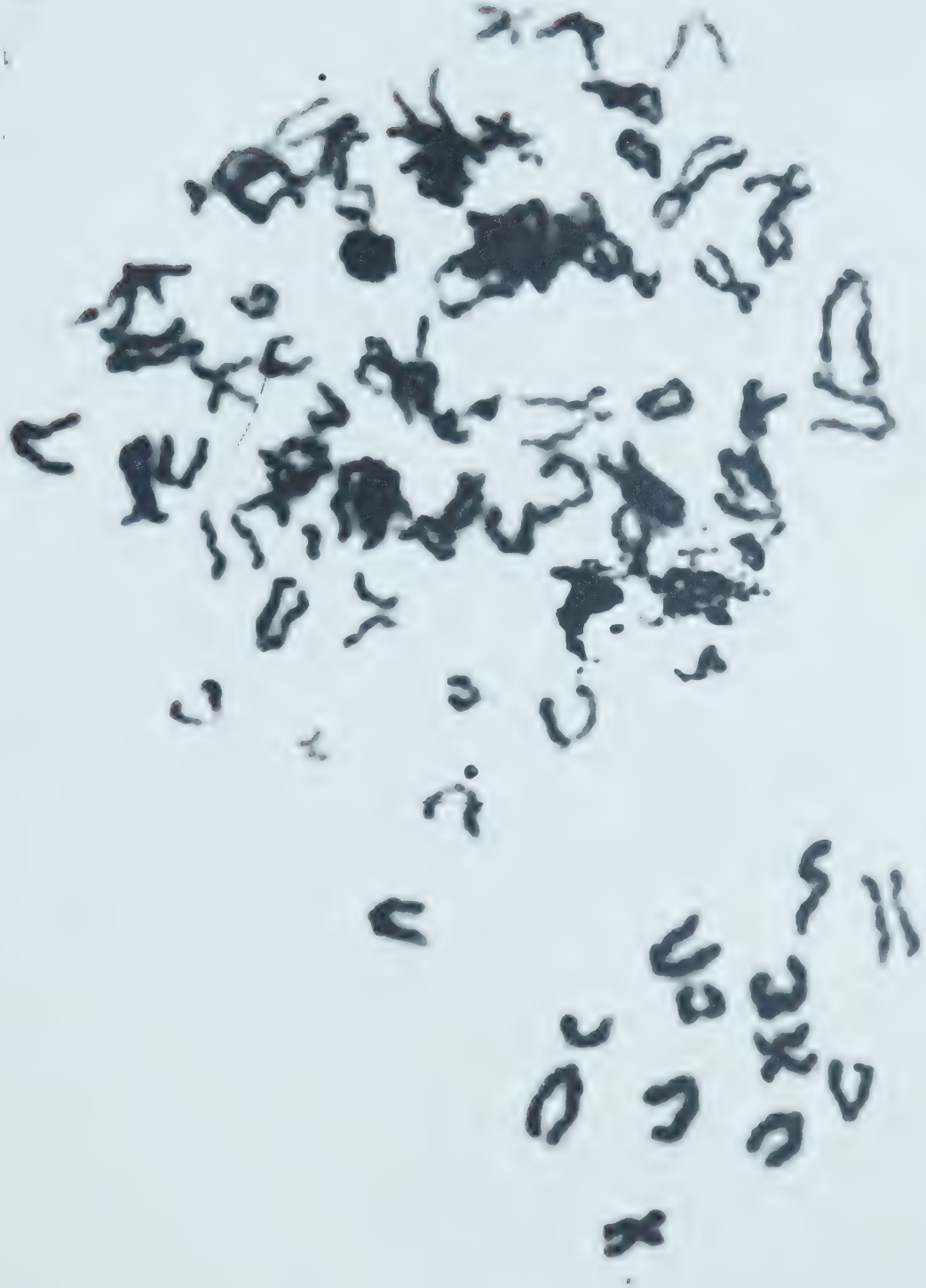
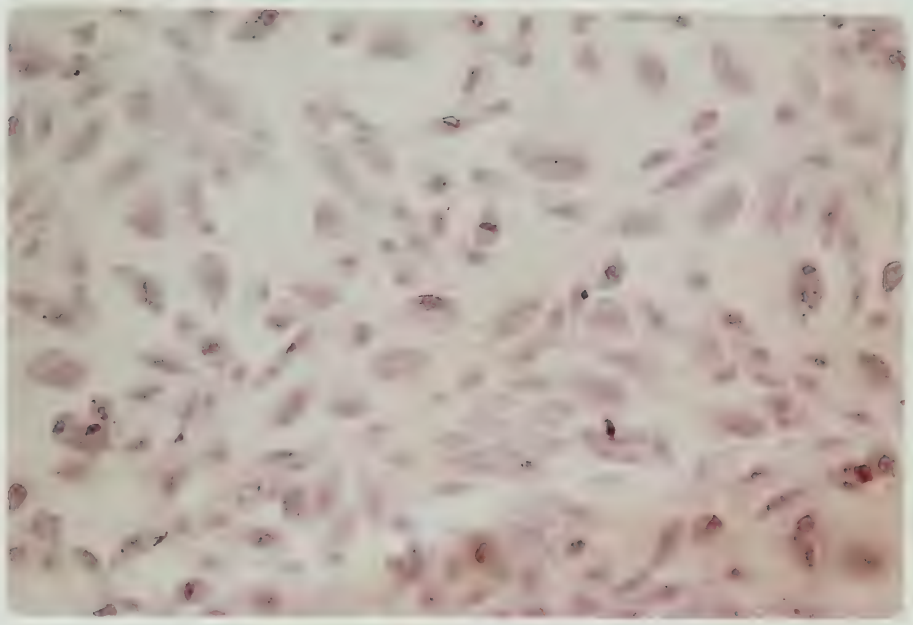


Figure 23. Hybrid "M" cell containing approximately 10-15 human-like chromosomes.

PLATE I. Human embryonic fibroblasts in monolayer culture. Wright's Stain.

PLATE II. (Same as above). Higher magnification showing a fibroblast in the process of cell division.



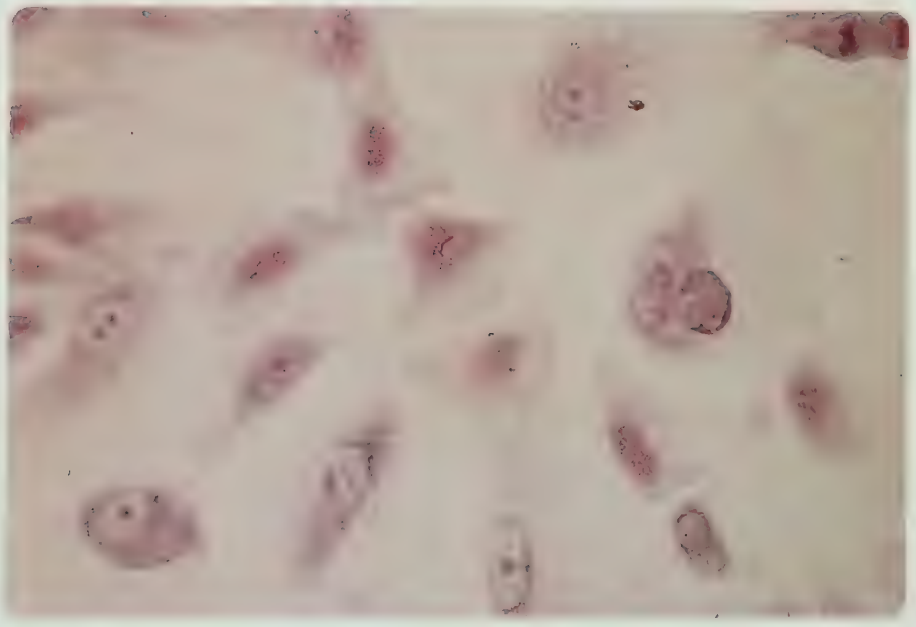
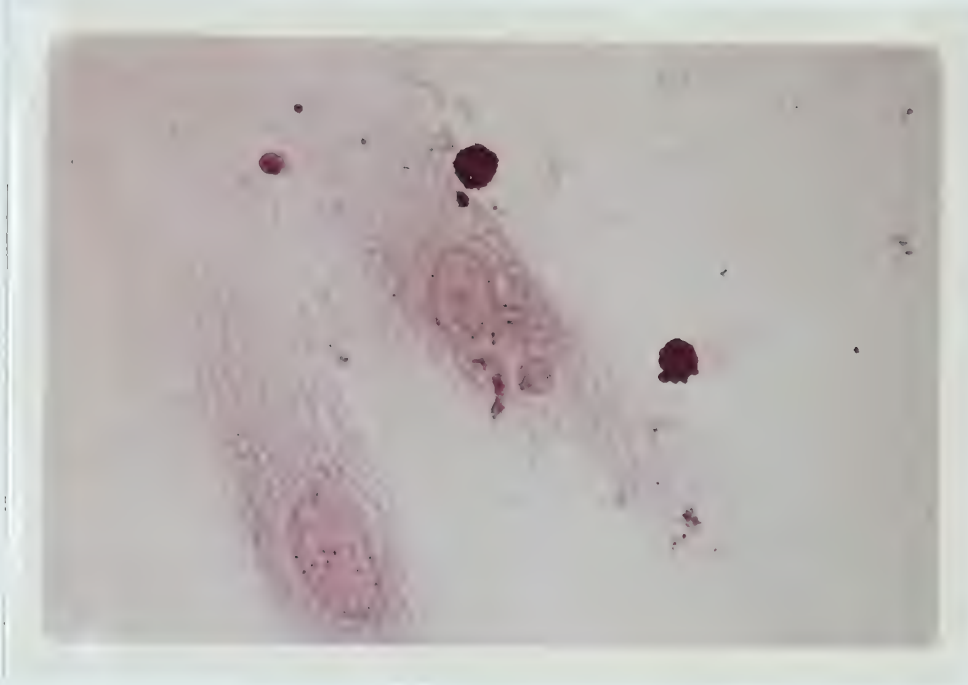


PLATE III. Heterokaryon containing a human fibroblast nucleus (large, oval and pale pink), and a mouse nucleus (small, round and dark-stained. Wright's Stain.

PLATE IV. Heterokaryon containing 2 human fibroblast nuclei and a number of mouse lymphoblast nuclei. Wright's stain.



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